STUDIES ON COHABITATION OF F-PRIME FACTORS IN ESCHERICHIA COLI K12

by

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EX BIBL. UNIV. EDINBURGEN

Thesis presented for the Degree of Doctor of Philosophy.

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November, 1971.

TO MY PARENTS

TO GIOCONDA

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ABSTRACT

The F-factor of *Escherichia coli* can exist either in the integrated state, in Hfr strains, or in an autonomous state, in F^+ or F-prime strains. Two different autonomous F-prime factors cannot replicate independently in the same cell, neither can an autonomous F-prime factor replicate in the presence of an integrated one.

This thesis is concerned with the incompatibility between autonomous F-prime factors in *Escherichia coli*. A number of F-prime factors were studied for their ability to cohabit, as determined by genetic criteria, in a multiply auxotrophic *recA* strain after triparental crosses using *recA* F-prime strains in pairwide combinations. None of the stable "double" strains isolated by this procedure turned out to be simple cases of cohabitation. The patterns of segregation and transfer of these "doubles" could not be explained by assuming a simple model of cohabitation between the F-prime factors involved in each pair.

Attempts were then made to isolate mutant F-prime factors defective in incompatibility. When strains harbouring either F*gal*$^+$ or F*his*$^+$ were mutagenized, mutants of this sort were not found. However when the recipient strain was mutagenized, a variant of this strain was isolated in which these two and even three different F-prime factors could replicate apparently autonomously. These F-prime factors could be cured and the resulting F^- strain could be reinfected with F*gal*$^+$ and F*his*$^+$ to give a new strain carrying both F-prime factors.

Finally a series of F-prime factors were isolated from crosses between an HfrH donor strain and an F^- *recA* recipient. These F-prime factors carried markers on one, or both sides of the integrated sex factor of the HfrH. Among these an F*ura*$^+$ *thr*$^+$ apparently coexisted stably with either F*gal*$^+$ or F*his*$^+$ but not with KLF4(F*thr*$^+$ *leu*$^+$ *pro*$^+$).

A. The discovery of F

The transfer of genetic material from one bacterial cell to another was discovered by Lederberg and Tatum (1946a,b). By growing a mixture of two doubly auxotrophic strains of Escherichia coli K12 and plating out heavy suspensions of the washed mating mixture on selective minimal agar they demonstrated the presence of prototrophs arising from this mating at a much higher frequency (10^{-6} - 10^{-7} of the parental bacterial input) than that expected from reversion alone. The possibility of these prototrophs arising from "cross-feeding" (syntrophism) was ruled out experimentally. The fact that no transfer of the "prototrophic forming activity" was obtained when cultures of either one of the parents were treated with cell-free filtrates or supernatants from the other suggested that this process was dependent upon direct cell-to-cell contacts between members of the two parental strains (Davis, 1950; Lederberg et al., 1951).

From experiments involving treatment of the parental cells with streptomycin and ultraviolet irradiation (Hayes, 1952a,b) it was evident that the parents do not play equivalent roles in conjugation, but that transfer of genetic material from one cell to another is unidirectional. It was found that the ability of a particular strain to act as a donor of genetic material depends upon the presence in its cells of an extrachromosomal and transmissible element which was termed F (Lederberg et al., 1952; Hayes, 1953a,b; Cavalli-Sforza et al., 1953). Cells carrying this fertility factor (F^+) are donors of genetic material (males), while those lacking it (F^-) are recipients (females). Crosses of the type $F^+ \times F^-$ are fertile, $F^+ \times F^+$ crosses are much less fertile and $F^- \times F^-$ crosses are sterile.

B. The autonomous state

The autonomous state of F in F^+ cells has been demonstrated using both genetic and physical techniques. That F is carried in an autonomous state by F^+ cells is shown by the fact that the introduction of a few F^+ cells into a culture of female cells brings about the spread of the F^+ character throughout the entire female population (Lederberg et al., 1952). The kinetics with which F can spread in this epidemic fashion has been taken to indicate that F can replicate faster than, and independently, from the bacterial chromosome (Lederberg, 1958; De Haan and Stouthamer, 1963). The physical autonomy of F has been demonstrated by showing that when present in a host cell of different DNA base composition (G-C content), F DNA can be easily separated from the chromosomal DNA by CsCl density gradient centrifugation (Falkow et al., 1964; Falkow and Citarella, 1965) (see next section).

More evidence for the extrachromosomal nature of F was the isolation of F^- cells in F^+ populations arising from spontaneous loss of F. Although in F^+ strains the spontaneous loss of F is a rare event, the frequency of F^- variants can be greatly increased by different treatments. Hirota (1956) found that elimination of F from F^+ populations could be enhanced by the presence of cobalt and nickel salts. He also found that the conversion to F^- was accomplished more efficiently by treatment with acridine dyes (Hirota, 1960). Under optimal conditions 100% of the descendants of F^+ cells incubated in the presence of acridine orange were F^-. Clowes et al., (1965) reported that F as well as other extrachromosomal elements could also be "cured" from thymineless mutants under conditions of thymine starvation. Sodium dodecyl sulphate (SDS) and urea have also been found to mediate the curing of F and other sex factors (Tomoeda et al., 1968, 1970; Inuzuka, et al., 1969).

(i) Structure of F

Evidence that F is a DNA-containing structure was first obtained from experiments measuring incorporation of radioisotopes. Lavallé and Jacob (1961) showed that when an F^+ strain grown in a medium containing radiophosphorus (^{32}P) was crossed with an unlabelled F^- strain the presence of the newly transferred F factors in the recipient declined with time at a rate proportional to the disintegration of the incorporated ^{32}P atoms. Driskell-Zamenhof and Adelberg (1963) confirmed these results and further demonstrated that when the donor (F^+) was grown in the presence of mitomycin no incorporation of ^{32}P occurs. Since mitomycin in the conditions used in their experiments inhibits the synthesis of DNA (but not of RNA) it was concluded that F consists of DNA. More evidence of the DNA nature of F was supplied by Herman and Forro (1964) who observed that the fraction of unlabelled females receiving F from F^+ donors with their DNA labelled with tritiated thymine prior to the mating was very close to the fraction of females receiving the labelled DNA. The value estimated for the size of F by these techniques was between 1.3 to 4.5% of the bacterial chromosome.

A more direct way of studying the nature and composition of F has been made possible by the discovery that it can be transferred from E.coli to Proteus mirabilis and Serratia marcescens despite the marked divergence in the G-C content in the DNA of these organisms (Falkow et al., 1964). Since the buoyant density of DNA is a function of base composition, the DNA of E.coli is easily separated by CsCl density gradient centrifugation from the DNA of P.mirabilis or S.marcescens. F DNA and the DNA of E.coli itself, have the same base composition (G-C content approximately 50%) and cannot be separated with this method (Falkow et al., 1964; Falkow and Citarella, 1965).

Falkow and Citarella (1965) isolated DNA representing two different F-prime factors (F13 and F15) (F factors carrying in addition chromosomal genes) which had been transferred from E.coli to S. marcescens. They estimated the size of F by hybridizing the DNA representing the different F-prime factors. Since these differ only in the chromosomal genes they carry it was assumed that only the homologous F segments would hybridize. In this way the size of F itself was estimated to be equivalent to 1.9% of the E.coli genome. Intergeneric transfer of genetic material has also been used to investigate the size and DNA composition of some other sex factors, such as: R factors (Watanabe, 1963; Falkow et al., 1966; Rownd et al., 1966), ColE1 (De Witt and Helinski, 1965) and some F-prime factors (Freifelder, 1968b).

Freifelder and Freifelder (1968a) devised a method for labelling sex factor DNA which consisted in using an F^+ carrying strain unable to incorporate exogenous radioactive thymine, as donor in matings with a female strain in which replication, as measured by thymine incorporation, was prevented by previous treatment with heavy doses of ultraviolet irradiation. Assuming that sex factor replication is autonomous, the only labelled DNA should be that synthesized by the sex factor during its replication in the recipient cells. Freifelder and Freifelder (1968b) compared the sedimentation velocity of the labelled sex factor DNA with that of phage λ DNA and estimated the molecular weight of F to be about 35×10^6 Daltons.

Electron microscopy observations of isolated F DNA as well as that from ColE1 (Roth and Helinski, 1967), ColVB (Hickson et al., 1967), $Flac^+$ (Freifelder, 1968a) and R factors (Nisioka et al., 1969) have confirmed that these particles consist of covalently closed circular double stranded DNA molecules. This circular structure has also been proposed on the basis of genetic studies (Broda, et al., 1964).

A further method for estimation of the molecular weight of F was used by Freifelder (1968b). It was based on the fact that X-ray irradiation introduces single-strand breaks into covalently closed circular DNA molecules and that such irradiation doses can be controlled to produce approximately one nick per circle. The "nicked circles" can be easily distinguished from covalent circles by their sedimentation coefficients in alkaline sucrose gradient centrifugation. Since the probability of conversion, with a given dose of X-ray irradiation, from closed circular DNA molecules to "nicked" circular is proportional to the molecular weight of the DNA molecules, he was able to estimate the molecular weight of F as 45×10^6 Daltons by comparison with the molecular weight of covalently closed circles of phage λ. This molecular weight represents a DNA content sufficient to code for some 40 to 60 proteins.

C. The integrated state

(i) Hfr strains

The role of F in conjugation was made clear by the independent discoveries of Cavalli-Sforza (1950) and Hayes (1953b) of new types of donor cells which were called Hfr (high frequency of recombination) and which had arisen in Lederberg's (1947) F^+ strain 58-161. These Hfr cells differed strikingly from the F^+ parent strain in two respects. Firstly, for some chromosomal markers the yield of recombinants was 10^3 to 10^4 times higher than in F^+ crosses, while with other markers the number of recombinants was the same in the two types of cross. Secondly, unlike recombinants from $F^+ \times F^-$ crosses, which were generally F^+, recombinants from Hfr x F^- crosses were usually F^-, rather than either Hfr or F^+ (Hayes 1953b). It was also found

that F was not lost in the conversion to Hfr since F^+ revertants could be found in Hfr populations.

The key to the understanding of the mechanism of chromosomal transfer by Hfr strains was provided by Wollman and Jacob (1955, 1958) and Hayes (1957). By interrupting the process of conjugation at different times they showed that a given Hfr gives rise to recombinants in a fixed sequence which is characteristic for each Hfr strain. They also found that in prolonged crosses, the earlier a recombinant appeared the higher the yield of that recombinant. They concluded that chromosomal transfer by Hfr strains is an oriented process which starts from a particular place on the chromosome, the "origin" of transfer, and during which spontaneous breaks occur so that recipient cells receive chromosomal segments all of which start at the same origin but are of unequal lengths (Jacob and Wollman 1955, 1958).

Jacob and Wollman (1956, 1957) in Hfr x F^- crosses showed that the orders of transfer by different Hfr strains isolated from the same F^+ strain are circular permutations of each other, and they concluded that the chromosome is in fact a closed circular structure. Furthermore, they observed that in such Hfr x F^- crosses some of the recipients which had inherited the chromosomal markers transferred last by those particular Hfr strains had also inherited the Hfr character. They therefore postulated that the Hfr state is a consequence of the integration of F into the host chromosome at one of several possible integration sites which define the "origin" particular to each strain. To explain the mechanism of chromosomal transfer by Hfr strains they proposed that the closed circular chromosome breaks at the site of F insertion to form a linear structure in which F is located at the opposite end to the origin. Chromosome transfer proceeds sequentially from the

origin so that only those rare recipients which receive the whole chromosome are converted to the Hfr state. The observation (Hirota, 1960) that F is not eliminated from Hfr bacteria by acridine orange treatment, as it is from F^+ cells, supports the view that F, in Hfr strains, is integrated into the chromosome.

In subsequent sections we shall discuss how the Campbell model, which was devised to explain the establishment of lysogeny, can also be applied to the integration of F. The actual evidence for the integration of F into the chromosome has been provided by the study of F-prime factors, properties of which will be discussed later.

(ii) The fertility of F^+ populations

It was proposed (Jacob and Wollman, 1956) that the basis for F^+ fertility is that in any F^+ culture there is a minority of cells which are Hfr. Since F can become integrated at different sites in the chromosome, in any given F^+ population there would be a heterogeneous population of Hfr cells with different origins and directions of transfer, so that the F^+ population as a whole would transfer all chromosomal markers at approximately the same low frequency.

Jacob and Wollman (1956, 1961) applied the fluctuation test of Luria and Delbrück (1943) to an F^+ population. As predicted by this hypothesis, they obtained subcultures with greatly enhanced fertility, from which they were subsequently able to isolate Hfr strains. However Broda (1967) and Curtiss and Stallions (1969) found that the frequency of stable Hfr clones in an F^+ population could not account for all recombinants formed in $F^+ \times F^-$ crosses. Moreover Curtiss and Renshaw (1965, 1969) described two types of F^+ strains, one which gave rise to stable Hfr derivatives (Type I), while the other

seldom did (Type II). Curtiss and Stallions (1969) presented evidence that in F^+ (Type I) x F^- crosses only 20% of the recombinants formed are due to stable Hfr clones. They postulated that the rest of the recombinants (80% and 100% in the case of Type I and Type II strains respectively) are due either to unstable Hfr clones or to a transfer mechanism which does not require F integration.

(iii) The concepts of episome and plasmid

F is an extrachromosomal self-transferring genetic element which cannot exist in a free state outside its host cell. In the autonomous state F behaves as an autonomous extrachromosomal element which can be eliminated by treatment with acridine orange (Hirota, 1960). In the integrated state F becomes physically associated with the chromosome (Jacob and Wollman, 1957, 1961) and replicates with it (Berg and Caro, 1967; Abe and Tomizawa, 1967; Wolf et al., 1968). In 1958 Jacob and Wollman introduced the concept of "episome" or "episomic element" to describe extrachromosomal genetic elements which may exist in these two alternative states. The information which led to the introduction of this term was obtained mainly from studies on the bacteriophage lambda (λ) in which the integrated and autonomous states are seen in the lysogenic (prophage) and lytic (vegetative growth) states respectively. Unlike F, phage λ , like other temperate bacteriophages, can be found free of its host cell.

Numerous other extrachromosomal genetic elements have been described in E.coli and other bacteria. Among them are the bacteriocin factors, which determine bacteriocin production (see Fredericq, 1957; Ivanovics, 1962; Reeves, 1965; Nomura, 1967), resistance transfer factors (R factors), which

confer upon the host cell resistance to one or more antibacterial drugs (see Watanabe and Fukasawa, 1961; Watanabe, 1963; Meynell et al., 1968) and some other less studied transfer factors (see Falkow et al., 1961; Falkow and Baron, 1962; Wohlhieter et al., 1964; Smith and Halls, 1967, 1968). Since for many of these extrachromosomal elements an integrated state has not been demonstrated, the term "plasmid" introduced by Lederberg et al. (1952) is widely preferred as a genetic term for all extrachromosomal hereditary units (see Falkow et al., 1967; Novick, 1969; Hayes, 1969).

D. F-prime factors

(i) The discovery of F-prime factors

In 1960 Adelberg and Burns reported the isolation from an Hfr strain (Hfr P4x) of a variant harbouring a new type of F factor which besides mediating its own transfer, like an autonomous F factor, also transferred a segment of the bacterial chromosome with high efficiency, like an integrated F factor. The progeny from such matings were themselves high frequency donors both for the F factor and for the chromosomal segment transferred by the parent strain. This variant F factor was presumed to be a hybrid autonomous sex factor in which F was covalently linked to a segment of the host chromosome. Many such F factors, which are referred to as F-prime (F') factors, have now been isolated.

In the same paper Adelberg and Burns showed that acridine orange treatment of the strain in which this variant F factor arose (P4x-1) resulted in the formation of F^- cells in which a wild type F subsequently introduced gave rise to a high frequency of chromosome transfer with the same orientation as that of the original Hfr strain, P4x. They suggested that in the event in which the Hfr strain gave rise to an autonomous sex factor, there occurred a genetic

exchange by which a piece of F remained in the host chromosome, forming a "sex factor affinity locus" (sfa) providing a region of homology for the attachment of any autonomous sex factor. The presence in the chromosome of this sfa locus has been supported by the finding that it is cotransducible with the lac marker (Driskell-Zamenhof, 1964). Another sfa locus, obtained from a different Hfr strain (W2817) located at a different site in the chromosome has also been described (Richter, 1957, 1961).

(ii) Integration of F and F-prime factors into the chromosome

In 1962 Campbell proposed a model to account for the insertion of prophage λ into the host chromosome which is also applicable to the integration of F giving rise to Hfr cells. Campbell's model was based in the assumption that prior to integration an episome is circular. He pointed out that a single reciprocal crossover event between such a circular prophage chromosome and the bacterial chromosome would linearise the phage chromosome and simultaneously insert it in the bacterial chromosome. Extensive evidence in support of this model exists for prophages λ and ø80, and has been reviewed in detail by Scaife (1967).

It seems very likely that the Campbell model also holds for the integration of F and F-prime factors and also for the mechanism by which F-prime factors mediate chromosome transfer. The circularity of F and F-prime factors has been demonstrated by physico-chemical and electron-microscopical methods (Freifelder, 1968a, 1968b).

The integration of F may require certain homology with the chromosome since its integration has been seen to occur only in a limited number of sites in the bacterial chromosome (Matney et al., 1964; Boyer, 1966; Scaife 1966;

Broda, 1967). The presence of homologous regions, allowing precisely such crossover events, appears to be the basis for F-prime mediated chromosome transfer (Scaife and Gross, 1963). Further evidence comes from the observation that such chromosome transfer is greatly reduced when the homologous region has been deleted from the host chromosome (Pittard and Ramakrishnan, 1964; Scaife and Pekhov, 1964; Cuzin and Jacob, 1967a).

The isolation of recombination-deficient (Rec^-) strains of E.coli (Clark and Margulies, 1965) has suggested that integration of F-prime factors may involve two different mechanisms. On the one hand recombination between the homologous regions of the chromosome segment carried by the F-prime factor and the host chromosome may involve the same enzymes as those promoting recombination between chromosomes, since integration of a F-prime factor into a chromosome with which it has homology occurs very much more frequently in a $recA^+$ strain than in a recA strain (Wilkins, 1969; DeVries and Maas, 1971). On the other hand, in the absence of homology between chromosome and episome, Broda and Meacock (1971) demonstrated that integration of an $F_{ts}lac^+$ into a recA strain carrying a large lac pro deletion occurs at a frequency comparable to that of the $recA^+$ analogue. Recombination between two F-prime factors in a recA strain has also been described (Press et al., 1971). These observations strongly suggest that integration of F may proceed by a mechanism other than the Rec system. Recombinational events independent of the Rec system are known; examples are the integration of λ (Gottesman and Yarmolinsky, 1968), the deletion of segments of the bacterial chromosome and the formation of low frequency specialized transducing phage particles (Franklin, 1967; Inselburg, 1967). A possible alternative mechanism for the integration of F is that F may carry gene(s) which may provide the necessary integration

enzymes. Such genes would be analogous to the int gene(s) of phage λ which are responsible for its integration into the host chromosome (Gingery and Echols, 1967).

Evidence of the integrated state of F comes from studies on F13 which is a circular structure and is known to carry markers from both sides of the integrated F confirming that F is integrated into the continuity of the chromosome. Also, deletions lacking both F and chromosomal functions have been isolated from Hfr strains (Ippen, Achtman and Willetts, in preparation) again indicating that F is continuous with the chromosome.

(iii) The formation of F-prime factors

Campbell (1962) proposed that strains lysogenic for λ gave rise to gal$^+$ transducing phage λ particles (λdg) as a result of excision of λ and gal$^+$ by a reciprocal genetic exchange between sites different from those involved in the integration event. It has been proposed (Broda et al., 1964; Scaife, 1967) that F-prime factors arise by analogous recombination events involving sites different from those involved in F integration. The Campbell model also provides an explanation for the origin of sfa loci, since, in the formation of F-prime factors, F gene(s) might sometimes be left in the host chromosome during the transition of F from the integrated to the autonomous state.

The first method for the isolation of F-prime factors, involved selection for the early entry of a very late Hfr marker in an Hfr x F$^-$ cross (Jacob and Adelberg, 1959; Hirota and Sneath, 1961; Pittard et al., 1963). Recently a very efficient method of isolating F-prime factors carrying early and/or late markers has been described (Low, 1968). This method takes advantage of the fact that in a recA recipient the normal integration of Hfr markers is

eliminated and therefore is possible to select for F-prime factors. Using this method it has also been possible to isolate F-prime factors defective in transfer; these presumably have deletions at one or other end of the integrated F (Low, 1968). These deletion mutants may be very useful in the mapping of F. The isolation of F-prime factors carrying early, late and both early and late markers, some of which appear to lack F functions, is described in section III of this thesis.

E. Functions of F

(i) Properties associated with the transfer function

The presence of an F factor in a bacterium can be recognized by two main effects. Firstly, the cell acquires the ability to act as a donor of genetic material in matings, and secondly, the presence of F results in the appearance of specialized filamentous surface appendages which have been termed F-pili (Brinton et al., 1964) to distinguish them from other more common forms of pili (fimbriae) found on both male and female cells. F-pili can be distinguished in the electron microscope from other types of pili and from flagella, and by the fact that F-pili act as the adsorption sites for certain bacteriophages which can therefore infect male but not female strains of E.coli (Crawford and Gesteland, 1964).

Two classes of F-specific phages have been described: members of one class are icosahedral and contain ribonucleic acid (RNA). Commonly used phages included in this class are f2 (Loeb, 1960), M12 (Hofschneider, 1963), MS2 (Clark, cited by Strauss and Sinsheimer, 1963) and Qβ (Watanabe, 1964). The second class are filamentous phages which contain single-stranded DNA; to this class belong f1 (Loeb, 1960), M13 (Hofschneider, 1963) and fd (Marvin

and Hoffman-Berling, 1963). RNA-containing phages attach to the side of the F-pilus (Brinton et al., 1964; Crawford and Gesteland, 1964), whereas DNA-containing phages attach to the tip (Caro and Schnös, 1966; Hoffman-Berling et al., 1966). The multiplication of the filamentous phages has the unusual feature of altering the metabolism of the host cell without immediately lysing the cell (Hofschneider and Preuss, 1963).

The conclusion that the synthesis of F-pili in E.coli is genetically controlled by the F factor is based on the fact that when an F^+ cell is cured of the F factor by treatment with acridine orange the cell loses the ability to produce F-pili and plate F-specific phage (Brinton et al., 1964). These authors also found that when F was restored by conjugation the newly made F^+ cells regained the ability to make F-pili and give plaques with an F-specific phage. Also mutants of F unable to produce pili have been isolated (Cuzin and Jacob, 1967a; Ohtsubo et al., 1970; Achtman et al., 1971).

Ørskov and Ørskov (1960) found that F^+ as well as Hfr strains of E.coli possess a specific antigen termed f^+ which is not detected in F^- cells. Male bacteria give a positive agglutination reaction with anti-f^+ serum. This antigen has been identified as the F-pilus and the agglutination reaction is inhibited when an F-specific phage is mixed with the male culture before the addition of anti-f^+ serum (Ishibashi, 1967).

Intact F-pili are essential for conjugation. Removal of F-pili by vigorous blending of a male population prior to a mating reduce the efficiency of recombinant formation (Brinton, 1965). Also male bacteria cannot act as donors if they fail to make F-pili (Hirota et al., 1966) or if their tips are blocked by F-specific phages (Ippen and Valentine, 1967). The precise role of the F-pilus in conjugation has not been yet fully established

but it has been suggested that F-pili are necessary for specific attachment in the coupling of mating cells (Brinton et al., 1964). These authors have also suggested that the F-pilus may serve as the conjugation tube through which F DNA and chromosomal DNA passes during the mating process.

Mutants of F-prime factors defective in their transfer ability (Tra^-) have been isolated from F-prime and Hfr strains of E.coli (Cuzin and Jacob, 1967a; Walker and Pittard, 1969; Ohtsubo, 1970; Ohtsubo et al., 1970; Achtman et al., 1971). Ohtsubo et al (1970) isolated Tra^- mutants of F from $Fgal^+$ and Hfr strains. These were assigned by these authors to seven cistrons on the basis of complementation tests involving the use of wild type and defective R factors (R-100-1). Achtman et al (1970) isolated many Tra^- mutants of an $Flac^+$ strain which have been assigned to eleven cistrons (traA to traK) from complementation tests in temporary heterozygotes carrying two mutant $Flac^+$ episomes. With the exception of traI, traG and traD which are probably involved in the metabolism of DNA during conjugation, these genes seem to be concerned with the formation of F-pili. TraJ seems to represent a regulatory gene involved with the control of transfer (Willetts, 1971; Finnegan and Willetts, 1971).

Crosses in which exponentially grown male cells are used as recipients have about 0.3% of the efficiency of the corresponding crosses using a female recipient; this inability to act as a recipient has been termed "surface exclusion." It does not seem to be dependent on the presence of F-pili in the male cell used as recipient since mutants of F unable to make pili show surface exclusion (Achtman et al., 1971). Also crosses between an F-prime donor strain and a DNA-less minicell derived from a F^+ parent, lacking F DNA and F-pili, are still subject to surface exclusion (Cohen et al., 1968).

Surface exclusion can be abolished by converting male recipients into phenotypic females (F^- phenocopies) which act as poor donors, by growing to stationary phase with vigorous aeration (Lederberg et al., 1952) or by treatment with periodate (Sneath and Lederberg, 1961).

(ii) Restriction of female specific phages

The presence of the F-factor can also be detected by its ability to inhibit the multiplication of a class of bacteriophages which have been grouped under the generic name of female-specific phages. Included in this class are phages ØI (Dettori et al., 1961), T3 (Schell et al., 1963) T7 (Mäkela et al., 1964), W31 (Watanabe and Okada, 1964), ØII (Cuzin, 1965) and tau (Hakura et al., 1964). These phages, are characterized by their ability to form large plaques when assayed on female cells, and smaller plaques at a reduced frequency on male cells. These phages adsorb equally well to male and female cells but fail to multiply in cells harbouring an F-factor. It has been proposed that the inability of phages ØII and T7 to multiply on male cells is due to the fact that F prevents transcription and translation of some of the infecting phage genome (see Linial and Malamy, 1970; Morrison and Malamy, 1971).

(iii) Replication of F

The genetic information of E.coli K12 is contained in a sigle, continuous DNA molecule, the chromosome (Jacob and Wollman, 1956). Genetic analysis (Jacob and Wollman, 1961) and autoradiography (Cairns, 1963) have shown that the chromosome is circular. Replication of the chromosome is semiconservative (Meselson and Stahl, 1958). On the basis of autoradiographic

experiments Cairns (1963) proposed that chromosome replication begins at a single point and proceeds in one direction along its length. However whether the E.coli chromosome has only one or several alternative origins of replication and also whether or not chromosome replication proceeds in only one direction is unclear.

Some authors have given evidence that in E.coli chromosome replication starts at a single point and proceeds unidirectionally (Bonhoeffer and Gierer, 1963; Lark et al., 1963; Lark, 1966). However this unique starting point of chromosome replication has been genetically defined at different sites in different strains (Abe and Tomizawa, 1967; Berg and Caro, 1967; Caro and Berg, 1968; Wolf et al., 1968; Yahara, 1971). Recent evidence suggests that replication of the chromosome, although starting at a single point, may proceed in both directions (Masters and Broda, 1971; Yahara, 1971).

When F is autonomous its replication is inhibited by treatment with acridine orange, which does not seem to affect chromosome replication at the concentrations used (Hirota, 1960). This curing was not due to a selective enrichment of the rare spontaneous F^- cells in the F^+ population but to the failure of F to replicate (see also Stouthamer et al., 1963; Hohn and Korn, 1969; Yamagata and Uchida, 1969). The independent replication of F in the autonomous state has also been demonstrated by the isolation of thermosensitive mutants affecting F replication without affecting that of the chromosome (Jacob et al., 1963).

In Hfr strains, although functions affecting transfer are fully expressed, F seems to be replicated passively as part of the chromosome, as suggested by the fact that F in the integrated state is not affected by acridine orange treatment. Also the position and orientation of F in this state, at least in E.coli, seem to have no effect on the origin and direction of chromosome

replication, confirming that replication of F in the integrated state seems to be under the control of the chromosome (Abe and Tomizawa, 1967; Berg and Caro, 1967; Wolf et al., 1968; Caro and Berg, 1969; Masters and Broda, 1971). However an earlier report (Nagata, 1963) suggested that in several Hfr strains of E.coli replication proceeds from the point at which the sex factor is integrated.

Jacob and Brenner (1963) (see also Jacob et al., 1963) proposed that structures such as the chromosome as well as extrachromosomal elements such as F, which are able to replicate as a unit should be termed "replicons". These authors postulated a replication mechanism based on the presence of regulatory elements analogous to those operating in the regulation of protein synthesis. According to their hypothesis each replicon would carry two specific determinants; an operator of replication termed the "replicator" on which a diffusible gene product, the "initiator", acts to trigger replication. These two elements would be replicon-specific in the sense that an initiator produced by a given replicon would recognize the replicator of that replicon but not those of other replicons.

On this model the sex factor in the autonomous state (F^+, F') would produce its specific initiator which would then act upon the F replicator and determine its replication independently of that of the bacterial chromosome. When F is integrated in the Hfr state its replication system would become in some way non-functional and the episome would be replicated under the control of the chromosome.

A prediction of this model is that for any replicon (F as well as the chromosome) it should be possible to isolate mutants unable to multiply independently. In fact this prediction has been fulfilled. Jacob et al (1963)

isolated thermosensitive mutants of F*lac*$^+$ unable to replicate at high temperature. These mutants with plasmid-linked mutations (F_{ts62}*lac*$^+$, F_{ts114}*lac*$^+$) could only replicate at the non-permissive temperature when the F*lac*$^+$ was integrated into the host chromosome and presumably replicated as part of it. Also Nishimura *et al* (1971) have observed that a temperature sensitive mutation (T46) of the chromosome affecting the initiation of its replication is not complemented by an autonomous F factor; however when temperature-resistant derivatives of such an F^+T46 strain were isolated, it was found that in many of them the sex factor had become integrated. The observation that the chromosome can replicate at the non-permissive temperature and that these Hfr strains are sensitive to acridine orange treatment suggests that in these strains chromosome replication is under the control of F.

Under optimum conditions the transfer of F from an F^+ cell (Jacob and Wollman, 1955) starts 4 minutes after the mating is initiated. To account for this rapid transfer of F in matings and for the regular distribution of daughter chromosomes and plasmids during cell division, Jacob *et al* (1963) have suggested that replicons are attached to the cell membrane, and that during replication the two copies of each replicon remain attached to it. After replication is completed, the newly made replicons move apart from each other as septum formation between them progresses.

From experiments involving the use of radioisotopes and temperature-sensitive F*lac*$^+$ mutants, Cuzin and Jacob (1967c) found that upon growth at high temperature, the F*lac*$^+$ particles remained associated for at least seven or eight generations with the same copy of the chromosome. A similar observation has been made from studies on segregation of the F-prime factor after acridine orange treatment (Hohn and Korn, 1969). The simplest mechanism for such a precise segregation mechanism would appear to be a membrane model of the type suggested by Jacob *et al* (1963).

This membrane attachment hypothesis has been supported by the observation that the nuclear bodies of B-subtilis, are always attached to one or two mesosomes (Ryter and Jacob, 1964, 1966); also biochemical studies (Ganesan and Lederberg, 1965) indicates that newly replicated DNA is associated with the membrane (see also Knippers and Sinsheimer, 1968; Standenbauer, 1971). Additional evidence for the role of the membrane in replication has come from the study of certain mutants which are defective in DNA synthesis, and which have been found to have altered membrane properties (Shapiro et al., 1970; Siccardi et al., 1971).

In the autonomous state the replication of F and some other replicons in E.coli is regulated so that there is only one copy of a given replicon per chromosome (Jacob and Monod, 1961). Jacob et al (1963) have suggested that this is because the number of the necessary membrane attachment sites specific to each type of replicon is limited to a few per cell. The saturation of this small number of attachment sites would also account for the phenomenon of "incompatibility", that is, the inability of two closely related sex factors to coexit stably.

An alternative model for the control of replication has been proposed by Pritchard et al (1969); it states that there is an inhibitor of initiation which is transcribed only at the time at which the chromosome is replicated. During cell growth the inhibitor is diluted out and its concentration falls to a critical value, triggering the initiator so start a new cycle of replication. Again a new pulse of inhibitor will be produced and further cell growth will again be necessary before a new replication cycle starts.

To account for the role of F in chromosome transfer Jacob et al (1963) have proposed that while vegetative replication of F in the integrated state is under the control of the chromosome, genetic transfer is due to the F replication

system. These authors based this proposal on the observation that treatment with acridine orange inhibits the initiation of the transfer of genetic material but does not affect chromosome transfer once it has been initiated (see also Cuzin and Jacob, 1966). Jacob et al (1963) have proposed that DNA synthesis is required in the donor during transfer and that one old strand and one newly replicated strand are driven into the recipient. However further experiments using isotopic labelling techniques to measure DNA synthesis and transfer have provided evidence incompatible with the Jacob et al (1963) model (see Curtiss, 1969, for review on this subject), and demonstrated that only one strand of donor DNA is transferred to recipient cells (Herman and Forro, 1964; Ptashne, 1965; Gross and Caro, 1966). This unique strand of the donor DNA has been identified as that with a leading 5' end; the complementary strand is synthesized in the recipient (Ohki and Tomizawa, 1968; Rupp and Ihler, 1968; Vapnek and Rupp, 1970).

(iv) The Mechanism of incompatibility

Jacob et al (1963), in their membrane-attachment-site model of regulation of DNA replication, suggested a mechanism of control which accounts for many of the properties of extrachromosomal elements. Since incompatibility is a phenomenon observed between closely related plasmids, this model also predicts that these attachment sites must be replicon specific. Thus plasmids belonging to different "incompatibility sets" would coexist because they would attach to different membrane-sites, whereas plasmids belonging to the same "incompatibility set" would compete for the same attachment site (Novick, 1969). The commonly observed fact that a superinfecting F-prime factor can displace a resident one very efficiently suggests that on this model the attachment of the F factor is not very firm. However this model does not account in a simple fashion for the failure of a superinfecting F factor to replicate stably

in an Hfr strain. If upon integration of the F factor into the host chromosome, its replication is, as is thought, under the control of the chromosomal replication system, the F attachment site should become free and a superinfecting F factor should therefore be able to attach to it.

Pritchard et al (1969) have proposed an alternative model for the control of replication based upon a cytoplasmic repressor analogous in action to that of phage λ. It is assumed that the proposed F-specific repressor, at a sufficiently high concentration prevents replication of both the integrated F and the autonomous F'-factor. If during growth both the superinfecting and the resident F-factors produce repressor this will reach a critical amount resulting in the inhibition of replication of one or other of the F-factors, so that the two F'-factors have equal probabilities of remaining in the cell. These two models need not be mutually exclusive, and the possibility that both mechanism can act together should be kept in mind.

Although E.coli cells normally harbour only one copy of F or of a given plasmid per chromosome in the autonomous state, some R-factors when transferred into P.mirabilis are present in about ten copies per chromosome in exponential phase and about sixty copies in stationary phase. This "relaxed" control of R factor replication in P.mirabilis does not appear to be a general phenomenon for episomes, since there is probably only one copy of an F'-factor per chromosome in this species (Rownd, 1969). Subsequent studies have revealed that certain R-factors in P.mirabilis exist in a multicopy pool from which individual copies are apparently selected at random for replication during the bacterial division cycle (Kasamatsu and Rownd, 1970).

(v) Incompatibility

In spite of the fact that F in the autonomous state replicates independently from the host chromosomes, its segregation and replication in E.coli is controlled in such a way that only one or two copies of F are associated with a given chromosome. This conclusion is based on the observations of Jacob and Wellman (1961), and Revel (1965) that the amount of β-galactosidase produced by an F*lac*$^+$/*lac* merodiploid was about twice that made under the same experimental conditions by a haploid *lac*$^+$ strain. On the assumption that a chromosome carries only one copy of this gene, these authors concluded that the merodiploid F*lac*$^+$/*lac*$^-$ may contain one or two functional copies of the F*lac*$^+$ per chromosome.

The control mechanism which limits the presence of a F-prime factor in a cell to only one or two copies may be the same as the one that prevents the establishment of two different F factors in the same cell. Scaife and Gross (1962) demonstrated that although an F*lac*$^+$ could infect an F$^+$ strain the two episomes did not coexist and that they segregated upon growth to give mixed colonies containing both F$^+$ and F*lac*$^+$ cells. De Haan and Stouthamer (1963) showed that although F*lac*$^+$ could infect cells carrying an F*gal*$^+$ factor, the progeny showing the Lac$^+$Gal$^+$ phenotype were very unstable, segregating Lac$^+$Gal$^-$ and Lac$^-$Gal$^+$ cells. This incompatibility between F*gal*$^+$ and F*lac*$^+$ was also observed by Echols (1963). He found that all the Gal$^+$Lac$^+$ progeny that he isolated had arisen by recombination of the Lac$^+$ marker carried by the F*lac*$^+$ with the chromosome and that only the F*gal*$^+$ was harboured in the autonomous state. However by crossing two variants of the same F-prime factor, one carrying the wild type allele for lactose fermentation and a mutant allele for alkaline phosphatase, and the other F-prime factor carrying the reciprocal alleles, he was able to isolate clones in which these two F-prime factors did seem to coexist stably. Experiments on segregation and transfer suggested that both variants were

replicating autonomously rather than existing in a hybrid structure. However because of the Rec^+ nature of the strains used, exist the possibility that this case of compatibility may have resulted from a mixed population in which either episome might have become integrated into the host chromosome.

In the experiments on cohabitation between autonomous F-prime factors described so far, crosses were made between a F-prime donor strain and a recipient that also carried a free F-prime factor. In contrast, Cuzin and Jacob (1967b) crossed two F-prime strains simultaneously with an F^- recipient. In such triparental crosses in which F*gal*$^+$ and F*lac*$^+$ were used as donors in a mating with anF^- *lac gal*D strain, they were able to select for the simultaneous inheritance of the episomal markers from both F-prime donor strains. Among the progeny these authors isolated rare stable colonies showing the Gal^+Lac^+ phenotype. Both F*lac*$^+$ and F*gal*$^+$ seemed to replicate autonomously since they were transferred independently of each other and with high efficiency to a further recipient and were also cured independently by treatment with acridine orange. However the small amount of information given by these authors prevents the elimination of other possible explanations such as recombination of either episomal marker with the chromosome which could also account for this apparent lack of incompatibility, also these two F-prime factors might have coexisted unstably for only part of the time. In a triparental cross in which two variants of the same F-prime factor, an F*lac* y^+z^- and an F*lac* y^-z^+, were used as donors, they failed to observe stable cohabitation. It should be pointed out that these results are the opposite to those previously described by Echols (1963) in the sense that while in Jacob and Cuzin's experiments variants of the same F-prime factor appeared not to coexist and different F-prime factors apparently did, in Echol's experiments the opposite results were obtained.

Incompatibility between an autonomous and an integrated sex factor also occurs. Following a cross in which a Lac$^-$ Hfr strain was superinfected with F*lac*$^+$ Scaife and Gross (1962) showed that those Hfr recipients which had acquired the Lac$^+$ phenotype did not harbour the F*lac*$^+$ factor, but that the expression of this phenotype seemed to be due to recombination between the F*lac*$^+$ and the homologous *lac* segment of the bacterial chromosome. It was concluded that multiplication of the autonomous F*lac*$^+$ is inhibited in Hfr cells. Similar evidence for inhibition of multiplication of an autonomous F-prime factor in a Hfr cell has been obtained by other workers (Maas and Maas, 1962; Maas, 1963).

In a more detailed study Dubnau and Maas (1968) studied the fate of a superinfecting F*lac*$^+$ in a *lac*$^-$ Hfr recipient in the F$^-$ phenocopy state, and found that although there was no barrier to the entry of the F*lac*$^+$ its replication is inhibited, and that the only *lac*$^+$ cells in the progeny are those in which the *lac*$^+$ gene has been rescued by recombination with the chromosome. Very few *lac*$^+$ progeny were found when a *lac recA* Hfr strain was used as recipient. However, even in the absence of recombination the *lac* gene can still be expressed, since in anHfr *recA* recipient, β-galactosidase could be produced on induction. These authors showed that the ability of the *lac*$^+$ allele entering the *recA* Hfr strain to be expressed persists at about the same level for at least seven generations; it appears therefore that the incoming F*lac*$^+$ is diluted out during growth rather than being actively broken down.

Cuzin and Jacob (1967b) also made a cross between anF*lac*$^+$ and a *lac*$^-$ Hfr (AB311) strain and found stable clones which seem to replicate both the autonomous F*lac*$^+$ and the integrated F. However Maas and Goldschmidt (1969) also obtained such strains and demonstrated, since some of the cells in the

same culture transmit *lac*$^+$ on the chromosome whereas other transmit it on the episome, that the F*lac*$^+$ factor was in fact unstably recombined into the host chromosome. Such "double" strains could not be isolated from a *recA* Hfr strain. This conclusion is in fact also implicit in the earlier results of Dubnau and Maas (see above).

Having shown that the great majority of "double" strains were artefacts due to recombination, Maas and Goldschmidt (1969) proceeded to isolate rare clones in which the replication of an autonomous F-prime factor in a *recA* Hfr (AB311) recipient cell was not inhibited. This lack of incompatibility is probably due to a deletion in the integrated F since when cured of the autonomous F-prime factor the Hfr strain is transfer-defective and deficient in surface exclusion, as well as being able to allow the cohabitation of an autonomous F-prime factor (Maas and Goldschmidt, 1969). The presence of an integrated defective F in this strain was suggested since after infection with either F*lac*$^+$ or F*thr*$^+$ *leu*$^+$ the strain regain the ability to donate chromosomal markers with the same origin and orientation as the original Hfr strain from which it was derived. However the presence of a *sfa* locus in this mutant, having arisen as described by Adelberg and Burns (1960), might also explain these results. The *sfa* locus would provide a region of homology for the attachment of the incoming F-prime factor and its recombination, although by a mechanism other than the Rec system, would bring about reconstitution of the donor ability and transfer of the chromosome with the same origin and orientation of that of the original Hfr strain.

Palchoudhury and Iyer (1971a) have found that a *recA*$^+$ temperature-sensitive mutant strain (DNA-ts43) unable to replicate its chromosome when grown at high temperature, allows stable cohabitation of both F*lac*$^+$ and F*thr*$^+$ *leu*$^+$ at the permissive temperature. On the basis of genetic and physico-chemical evidence

these authors have suggested that the two F-prime factors replicate autonomously from each other and from the host chromosome. However this cohabitation may be only transient since, as it might be expected from the Rec^+ phenotype of the host cell, integration of the episomal markers into the chromosome should occur.

In a mating between two Hfr strains, in which the recipient was in the F^- phenocopy state, and in which there was selection for the inheritance of a late chromosomal marker from the donor Hfr, Clark (1963) was able to isolate a strain which harboured two F factors integrated in different sites in the chromosome. The replication of these F factors seems to be very stable and this "double" male transfers genetic material to recipients in the form of two independent linkage groups with the origins of transfer corresponding to those of the parent Hfr strains. Any given cell of this "double" male transfers one or the other linkage group but not both. Bastarrachea and Clark (1968) using this "double" male as the recipient in a mating with F30(<u>his</u>$^+$) isolated a "triple" male strain in which F30 did not replicate autonomously but seemed to have become integrated, although unstably in the chromosome. This again suggests that in F-prime merodiploids of a Hfr strain, the F-prime factor escapes inhibition of its replication by integration into the host chromosome.

We can summarize this information as follows: incompatibility does not represent a barrier to the entry of a superinfecting F-prime factor but comes into play only after the F-prime factor has been transferred. Incompatibility is expressed very soon after entry but the F-prime factor that is being segregated out remains intact for at least seven or eight generations without being replicated. The superinfecting F factor can escape inhibition of its replication either by displacing the resident F factor, when in autonomous

state, or by recombining with the chromosome. All cases of cohabitation which have been described are open to criticism. Rec^+ strains have been used in most of these studies and it is likely that these results may reflect recombination between the sex factor and the chromosome or between the sex factors themselves. Also the limited number of F-prime factors used does not exclude the possibility that other F-prime factors may show a different incompatibility behaviour. It is not possible, with the information provided by the studies so far described, to distinguish between the two models proposed to account for incompatibility (Jacob *et al.*, 1963; Pritchard *et al.*, 1969). No mutants have been isolated which are defective in this function and which could help to discriminate between these two models. Moreover no really convincing cases of cohabitation of an autonomous F-prime factor either with an integrated F factor, or with another autonomous F-prime factor, have been observed.

In order to obtain more information about incompatibility we have embarked upon further studies on cohabitation of F-prime factors. In these experiments only recombination deficient (*recA*) strains have been used, in order to minimize the complications caused by recombination. This thesis comprises three experimental sections. Section I describes a systematic study of cohabitation using several F-prime factors; none of the "double" progeny that were isolated and characterised proved to be simple cases of cohabitation. Section II deals with mutagenesis of two F-prime factors, as well as of the F^- recipient, followed by selection for strains carrying cohabiting F-prime factors. Whereas mutagenesis of the F-prime factors did not result in the isolation of strains allowing cohabitation between two F-prime factors, mutagenesis of the host strain allowed the isolation of a mutant in which two and even three different F-prime factors seem to coexist stably. Section III describes the isolation of a $Fura^+ thr^+$ which seems to coexist stably with either $Fgal^+$ or $Fhis^+$ but not with $Fthr^+ leu^+ pro^+$.

EXPERIMENTAL INVESTIGATIONS

MATERIALS AND METHODS

A. Bacterial strains

The properties of the bacterial strains used are described in Table 1. ED1308 and ED1314 were derived from JC4566 (F^- *trp*$_{am}$ *leu*$_{am}$ *his lac spc tsx*) (obtained from Dr. M. Achtman) (itself a derivative made in several steps from W3110) by way of the following intermediate steps:

1) Isolation of a Gal^- mutant after treatment of JC4566 with ultra-violet irradiation.

2) A Thy^- (50 µg./ml.) derivative was selected after treatment of the Gal^- strain with trimethoprim (Stacey and Simson, 1965).

3) A Mal^- derivative of the Gal^-Thy^- strain was selected among cells of this strain resistant to phage λv. This $Mal^-Gal^-Thy^-$ strain was then used as recipient in crosses with JC12 (Hfr *mal*$^+$ *met*) (Clark, 1963) and Met^- recombinants were selected among the Mal^+ progeny.

4) The $Gal^-Thy^-Met^-$ strain was used as a recipient in a mating with JC5088 (Hfr *thy*$^+$ *recA*) (Willetts and Clark, 1969) and $RecA^-$ recombinants were obtained among the Thy^+ progeny.

5) Both ED1308 and ED1314 are derivatives of one such Gal^-Met^- $RecA^-Thy^+$ strain. ED1308 was selected as a spontaneous Spc^r derivative and ED1314 is a Thy^- (50 µg./ml.) derivative obtained after treatment with trimethoprim.

ED2742 is a Gal^-(UV irradiation) and spontaneously Spc^r derivative of PA2004 (F^- *thr leu his pyrB lac str*) (obtained from Dr. E. Moody).

Table 14 presents a list of presumptive F-prime factors isolated in section III of this thesis together with some of their properties.

Strain No.	Relevant genotype: leu	his	recA	gal	lac	str	spc	other markers	F-prime factor(s)	Derivation
ED1314	-	-	-	-	-	R	S		-	See text
ED1308	-	-	-	-	-	R	R		-	See text
ED1221	-	+	-	+	-	S	R		-	See text
ED1229	-	+/+	-	+	-	S	R		F30(his$^+$)	derivative of ED1221
ED1232	-	+	-	+/+	-	S	R		F8 (gal$^+$)	derivative of ED1221
ED1283	-	+	-	+	+/-	S	R		F13(tsx$^+$ ade$^+$ lac$^+$)	derivative of ED1221
ED1287	-	+	-	+	+/-	S	R		F42(lac$^+$)	derivative of ED1221
ED1291	+/-	+	-	+	-	S	R		KLF1(thr$^+$ leu$^+$)	derivative of ED1221
ED1294	+/-	+	-	+	-	S	R		KLF4(thr leu pro$^+$)	See text
ED2601	-	+	-	+/-	-	R	S		F8(gal)	See text
ED2602	-	+/-	-	+/-	-	R	S		F8(gal$^+$)/F30(his$^+$)	See text
ED2604	-	+/-	-	+/-	-	R	S		F8(gal$^+$)/F30(his)	See text
ED2605	-	+	-	+/-	-	R	S		F8(gal$^+$)	See text
ED2606	-	+	-	+/-	-	R	S		F8(gal$^+$)	See text
ED2607	-	+	-	+/-	-	R	S		"	See text
ED2608	-	+	-	+/-	-	R	S		"	See text
ED2610	-	+	-	+/-	-	R	S		"	See text
ED2612	-	+	-	+/-	-	R	S		"	See text
ED2614	-	+	-	+/-	-	R	S		"	See text
ED2615	-	+	-	+/-	-	R	S		"	See text
ED2616	-	+	-	+/-	-	R	S		"	See text
ED2618	-	+/-	-	+/-	-	R	S		F8(gal$^+$)/F30(his$^+$)	See text
ED2620	-	+/-	-	+/-	-	R	S		" "	See text
ED1320	-	-	-	+/-	-	R	S		F8(gal$^+$)	ED1232 x ED1314
ED1323	-	+/-	-	-	-	R	S		F30(his$^+$)	ED1229 x ED1314
ED2713	-	+/-	-	-	-	R	S		F30(his$^+$)	AO curing of ED2620
ED2714	-	-	-	+/-	-	R	S		F8 (gal$^+$)	AO curing of ED2620
ED2745	-	-	-	-	-	R	S		-	AO curing of ED2620
ED2751	-	+/-	-	-	-	R	S		F30(his$^+$)	ED1229 x ED2745
ED2753	-	-	-	+/-	-	R	S		F8 (gal$^+$)	ED1232 x ED2745
ED2715	-	+/-	-	+/-	-	R	S		F8(gal$^+$)/F30(his$^+$)	ED1232 x ED2751

Strain No.	Relevant genotype: leu	his	recA	gal	lac	str	spc	other markers	F-prime factor(s)	Derivation
ED2716	-	+/-	-	+/-	-	R	S		F30(*his*$^+$)/F8(*gal*$^+$)	ED1229 x ED2714
ED2767	-	+/-	-	+/-	-	R	S		" "	ED1232 x ED2751
ED2775	-	+/-	-	+/-	-	R	S		" "	ED1229 x ED2753
ED2735	-	-	+	-	-	R	R			Rec$^+$ isogenic of ED1308
AB259	+	+	+	+	+	S	S			HfrH (ex Hfr 3000) (Taylor and Adelberg 1960)
KL132	-	-	-	-	-	R	S	*thr*$^-$ *pro* *pyrB*		B. Low (1968)
ED1490	-	-	-	-	-	-	-	" " "		Spontaneous Spc^r of KL132
ED2742	-	-	+	-	-	R	R	*thr*$^-$ *pyrB*		derivative of PA2004 (obtained from Dr.E. Moody) (see text)

Table 1. Bacterial strains.

The nomenclature used is that recommended by Demerec *et al.*, (1966) and Taylor and Trotter (1967).

Abbreviations used are: Leu = leucine; His = histidine; pro = proline; Thr = threonine; Ura = uracil; Rec = recombination:

+ = proficient, independent, or utilizing; - = deficient, dependent or nonutilizing

Str = streptomycin; Spc = spectinomycin. Resistance and sensitivity are denoted by R and S respectively.

B. F-prime factors

All F-prime factors used in this thesis, except those isolated in the course of this study (section III) were maintained in strain ED1221, a recA derivative of JC6554 ($F^- trp_{am}$ leu_{am} lac spc) (obtained from Dr. M. Achtman). These F-prime factors are the following:

F30(his$^+$) (Matney et al., 1966)

F8(gal$^+$) (Hirota and Sneath, 1961)

F42(lac$^+$) (Adelberg and Burns, 1960)

F13(tsx$^+$ purE$^+$ lac$^+$) (Hirota and Sneath, 1961)

KLF1 (thr$^+$ leu$^+$) (Low, 1968)

KLF4 (thr$^+$ leu$^+$ pro$^+$) (Low, 1968)

Strains were maintained at 4°C on nutrient L-agar or, in the case of merodiploid strains, on selective minimal agar to reduce spontaneous loss of the F-prime factor(s).

C. Bacteriophages

The male specific phage MS2 (Clark, cited by Strauss and Sinsheimer, 1963) and the female specific phage ØII (Cuzin, 1965) were used as a rapid means of checking whether a given strain was male or female. Phage stocks were prepared by a confluent plate lysis technique, sterilised with chloroform and stored at 4°C. They were titred by mixing 0.05 ml of an appropriate dilution of the phage lysate with 0.1 ml of an overnight culture of the strain to be tested in 2.5 ml of LC soft agar which was then poured on freshly made LC plates.

D. Media

Nutrient broth: the complete medium used was Luria (L) - broth (pH approx. 7.2), which contains 10 g. Bactotryptone, 5 g. Difco yeast extract and 10 g. NaCl per litre of water.

Minimal medium: a synthetic medium based upon M9 salt mixture in water; 3g.KH_2PO_4, 7g.Na_2HPO_4, 0.5g.NaCl, 1g.$NH_4Cl.7H_2O$ and 1mM.$MgSO_4$ per litre. Sugars were added to a final concentration of 0.2%, thiamine to 0.2 µg./ml. and any required L-amino acids to 20 µg./ml.

Nutrient and minimal hard and soft agar: were made by solidifying the appropriate liquid media with 1.5% and 0.7% Davis New Zealand Agar respectively.

Eosin-methylene blue medium: contained 42.4 g.Difco Bacto-casamino acids, 5.2 g.Difco Bacto-yeast extract, 27 g.NaCl, 10.4 g.KH_2PO_4 and 15 g.Davis agar per litre. After this had been autoclaved, sugars were added to a final concentration of 1%, eosin to 0.4 mg./ml. and methylene blue to 0.065 mg./ml.

LC top agar: was nutrient L soft agar containing $CaCl_2$ to a final concentration of 5 mM.

E. Chemicals

Aqueous solutions of streptomycin sulphate (Glaxo Laboratories Ltd., Greenford, England) and spectinomycin sulphate (a gift of Upjohn Company, Kalamazoo, Michigan, USA) were both sterilised by filtration. These antibiotics were used at final concentrations of 200 µg./ml. and 100 µg./ml. respectively.

N-methyl-N'nitro-N-nitrosoguanidine (NTG) was dissolved in M citrate buffer (pH 5.5) at a concentration of 0.15 mg./ml. and sterilized by filtration. It was used as a mutagen at a final concentration of 30 µg./ml.

F. Mating methods

All matings were performed in L-broth at 37°C. Overnight cultures in L-broth of the cells used in matings were diluted 1:100 in fresh L-broth and were incubated in conical flasks fitted with tube side arms; growth was followed as the optical density of the culture in a Klett colorimeter to approximately 2×10^8 cells per ml. The cultures were then mixed in a 100 ml. conical flask and the mixture, which never exceeded 10 ml. in volume, was incubated at 37°C without agitation for 30 minutes. At the end of the mating period 1 ml aliquots of mating mixtures were violently agitated on a "Whirli Mixer" for 1 minute to separate mating pairs, after which 0.1 ml samples of suitable dilutions and of the appropriated controls were spread on selective media. In some experiments, where indicated, stationary phase cultures (obtained by overnight growth with rapid shaking) of recipient strains were used; this procedure is known to produce F^- phenocopies of F^+, Hfr and F-prime strains (Lederberg et al., 1952).

In biparental crosses the proportion of donor to recipient cells was usually 1:5 but in some experiments, which will be specified, this proportion was changed. In order to find the best proportion of parents to be used in triparental crosses an experiment was performed in which the parental inputs in the mating mixture could be related to the efficiency of transfer per recipient cell of the episomal markers carried by the donor F-prime strains. From such experiments the proportion 5:5:1, donors to recipient, was selected as the most suitable.

In some experiments, tests for cross-feeding were made simultaneously with some triparental crosses. Here donor and recipient cells were grown in L-broth to exponential phase, and then mixed in the same proportions as in matings. Immediately after mixing, 0.1 ml samples were plated on selective agar. Controls for reversion were also made in all matings. Plate matings by replica plating were performed according to the procedure described by Clark and Margulies (1965).

G. Replica plating

When colonies containing merodiploid cells from mating experiments were to be scored for the presence of unselected markers, or when the stability of F-prime factors was tested, clones were patched with the help of sterile tooth-picks in a geometrical pattern onto minimal agar medium of the same composition as that on which they arose. These plates were incubated until the patches showed confluent growth and were then replica-plated on appropriate selective media by the method of Lederberg and Lederberg (1952).

H. Purification and Phenotypic tests

All new strains whether obtained from mating or after mutagenesis were purified by repeated single colony isolation on either nutrient agar or minimal agar of the same composition as that on which they first arose. After purification, single colonies were grown in appropriate selective liquid minimal media in order to check amino acid requirements, ability to ferment sugars and sensitivity or resistance to antibiotics.

The Rec phenotype was scored by testing the UV-sensitivity of patched clones as described by Clark and Margulies (1965). High sensitivity to UV-irradiation

is associated with recombination-deficiency (Clark and Margulies, 1965).

I. Curing

The procedure used was that described by Bastarrachea and Wiletts (1968). Inocula of about 500 cells each from an overnight culture of the merodiploid strain to be cured were diluted into a series of 2 ml aliquots of L-broth at pH 7.8 containing concentrations of acridine orange which varied between 5 µg./ml. and 50 µg./ml. (different concentrations were used in attempts to get some curing which in general was exceedingly poor, see below). The cultures were then incubated for about 16 hours in the dark with shaking. Curing by acridine orange was taken as a criterion for the autonomous state of the F-prime factor(s). However it must be mentioned that in all cases where this treatment was applied in this thesis, excepting that of Gal^+Lac^+ "doubles", curing was very poor, usually less than 1%. This poor efficiency of curing was not due to the Rec^- property of the strains tested since in recA strains other than those mentioned in this thesis, treated in the same conditions, their F-prime factors were cured with high efficiency.

J. Mutagenic treatment

Aliquots of 5 ml of an exponential culture in L-broth were centrifuged, washed and resuspended in the same volume of fresh L-broth. NTG was added to give a final concentration of 30 µg/ml. and the mixture was incubated at 37°C. After 30 minutes incubation this mixture was centrifuged and washed twice in buffer to remove the NTG. A sample of the washed cell suspension was diluted in fresh broth and further incubated to allow expression of the mutant phenotype before plating onto selective medium.

SECTION I

ISOLATION OF SPONTANEOUSLY ARISING STRAINS CARRYING GENES FROM TWO F-PRIME FACTORS

A. Introduction

The object of these experiments was to overcome two objections to those studies on cohabitation of F-prime factors that have been described so far. The first of them is that, recombination of the superinfecting F-prime factor with either the chromosome (as shown by Dubnau and Maas, 1968; Maas and Goldschmidt, 1969) or with the resident F-prime factor (Cuzin and Jacob, 1967b), complicates the interpretation of the results obtained in these studies. The use of recA strains allows a better study of the autonomous F-prime factors since their integration into the host chromosome and into each other due to the host recombination system is greatly reduced. It would therefore appear to make the selection of clones carrying two genuinely cohabiting F-prime factors more feasible. Even though some recombination occurs in a recA recipient (De Vries and Maas, 1971; Broda and Meacock, 1971; Press, et al., 1971) possibly due to a Rec-independent mechanism (Franklin, 1967; Inselburg, 1967), this level is extremely low and compatibility between two autonomous sex factors should be detected.

The second objection is that only the incompatibility of F*lac*$^+$ with F*gal*$^+$ or other genetically distinguishable F*lac*$^+$ factors has been studied when considering the cohabitation of two F-prime factors as distinct from F' - Hfr cohabitation. The possibility also exists that other F-prime factors may not show the same incompatibility behaviour as F*lac*$^+$ and F*gal*$^+$, and that incompatibility between autonomous F-prime factors may proceed by a different mechanism

to that observed between an autonomous F-prime factor and an integrated F. It has been considered that a more systematic study using several F-prime factors in pairwise combinations coupled with a simpler and more effective selective system was necessary in order to get more information on the mechanism of incompatibility. Therefore, different combinations of six F-prime factors were used. An F^- *recA* multiply auxotrophic strain in which the presence of any of two of these different F-prime factors could be simultaneously selected for was constructed and used as recipient in these studies.

In this thesis, studies on cohabitation between autonomous F-prime factors rather than between an autonomous and an integrated F (Hfr) has been chosen, since studies on F' - Hfr incompatibility are being carried out in another laboratory (Dubnau and Maas, 1968; Maas and Goldschmidt, 1969; Maas, 1970), and instead of using an F-prime strain in F^- phenocopy state as the recipient, the method of Cuzin and Jacob (1967b) in which two different F-prime strains were simultaneously crossed with an F^- strain has been followed. Cohabitation of F*lac*$^+$ and F*gal*$^+$ using this mating technique has been claimed by these authors.

B. Method

The strain used as recipient in these experiments was ED1314, an F^- *recA leu his lac gal met str* strain in which the episomal markers carried by the F-prime factors in the donor strains could be selected simultaneously. In order to simplify the matings, all F-prime factors used in this study were in the same strain, ED1221 (F^- *recA leu lac spc*). The F-prime factors used were: F8(ED1232), F30(ED1229), F42(ED1287), F13(ED1283), KLF1(ED1291) and KLF4(ED1294). Their episomal markers are described in Material and Methods.

ED1314 was mated in triparental crosses with two different F-prime strains simultaneously as described in Materials and Methods. After interruption of the mating, aliquots from a series of dilutions were plated onto selective minimal agar and/or onto EMB (when F*gal*$^+$ and F*lac*$^+$ were used). Streptomycin was used to kill the donors, and was also included in all plates on which subsequent purifications were performed. Since it was possible that colonies growing on selective minimal plates could arise by cross-feeding rather than by inheritance of episomal markers from the donor strains, tests for cross-feeding were carried out simultaneously with each mating. Also tests for reversion and crosses using each donor singly were performed as controls. Clones inheriting markers from both donor F-prime strains were selected in two different ways: one was to use selective minimal agar on which only that fraction of the progeny which had inherited episomal markers from both donor F-prime strains could grow, and the other was to select for only one marker and then by replica-plating on selective media look among those clones for the inheritance of the unselected marker.

Clones isolated in either way were tested for the inheritance of both episomal markers and purified as follows:

(a) From mating plates selecting for two markers: 20 single and well separated colonies were streaked out on nutrient agar containing streptomycin. After overnight incubation 20 single colonies of each were patched on non-selective minimal agar and then replica-plated onto the same selective minimal agar on which these colonies were originally isolated. Clones which showed the presence of both episomal markers were again streaked on nutrient agar and 20 single colonies of each were then patched and replica-plated as described. This purification procedure was repeated until no more or very low ($< 1\%$) segregation of either of the episomal markers

involved was observed. The frequency with which stable "double" colonies were isolated by this procedure depended upon the particular pair of F-prime factors involved. More information concerning the isolation of "doubles" with each pair of F-prime factors used, is given in each particular section.

(b) From mating plates selecting for only one marker: 100 well separated colonies were patched on minimal agar of the same composition used for selection, and were replica-plated onto selective minimal plates on which only cells which had inherited both episomal markers could grow. Any such clone was treated as in (a).

The criterion used for stability was absence of segregation: single purified colonies showing inheritance of episomal markers from both donor F-prime strains were grown overnight in L-broth and samples of these cultures were either plated onto nutrient L-agar and then replica-plated onto selective minimal agar, or, when segregation of a sugar marker was being tested, loopfuls of the cultures in L-broth were streaked out on the appropriate EMB media. A clone was termed a "stable double" when after this test only very low segregation ($<1\%$) of the markers involved was observed.

C. Results

The F-prime strains enumerated in (B) were used in pairwise combinations in triparental crosses as described above. A summary of the results obtained in this section may be seen in Table 2. In general, the yield of progeny of each type in triparental crosses is reduced to about 30 to 50% of that in control biparental crosses. When selection was for only one marker

Donor (a) F-prime factors		Markers selected (b)		phenotype and relative frequency of unpurified progeny (c)	acridine orange curing	donorability (d) of "double" strains
F*gal*$^+$; F*lac*$^+$	(e)	*lac*$^+$	(f)	Lac$^+$ = Gal$^+$ = Lac$^+$Gal$^+$	Gal$^-$Lac$^-$ (g)	Gal$^+$Lac$^-$; Gal$^-$Lac$^+$. No Lac$^+$Gal$^+$ (later only Gal$^+$). (h)
" "		*gal*$^+$	(f)	Gal$^+$ ≫ Lac$^+$ = Lac$^+$Gal$^+$	" "	
F*leu*$^+$; F*gal*$^+$	(i)	*leu*$^+$		Leu$^+$ > Gal$^+$ = Leu$^+$Gal$^+$	Leu$^+$Gal$^-$	Gal$^+$Leu$^-$; Gal$^+$Leu$^+$; no Leu$^+$Gal$^-$
" "		*gal*$^+$		Gal$^+$ ≫ Leu$^+$ = Leu$^+$Gal$^+$	" "	" " "
" "	(i)	*leu*$^+$ *gal*$^+$		Leu$^+$Gal$^+$	" "	" " "
F*leu*$^+$; F*his*$^+$		*leu*$^+$		Leu$^+$ > His$^+$ = Leu$^+$His$^+$	Leu$^+$His$^-$	His$^+$Leu$^-$; His$^+$Leu$^+$; no Leu$^+$His$^-$
" "		*his*$^+$		His$^+$ ≫ Leu$^+$ = Leu$^+$His$^+$	" "	" " "
" "		*leu*$^+$ *his*$^+$		Leu$^+$His$^+$	" "	" " "
F*lac*$^+$; F*his*$^+$	(e)	*lac*$^+$		Lac$^+$ > His$^+$ = Lac$^+$His$^+$	Lac$^+$His$^-$	His$^+$Lac$^-$; His$^+$Lac$^+$; no Lac$^+$His$^-$
" "		*his*$^+$		His$^+$ ≫ Lac = Lac His	" "	" " "
" "		*lac*$^+$ *his*$^+$		Lac$^+$His$^+$	" "	" " "
F*leu*$^+$; F*lac*$^+$	(j)	*leu*$^+$		see text		
" "		*lac*$^+$				
" "		*leu*$^+$ *lac*$^+$				
F*gal*$^+$; F*his*$^+$	(j)	*gal*$^+$		Gal$^+$ ≫ His$^+$ = Gal$^+$His$^+$	see text	
		his$^+$		His$^+$ ≫ Gal$^+$ = Gal$^+$His$^+$		
		gal$^+$ *his*$^+$		Gal$^+$His$^+$		

Table 2 Summary of results on the nature of "double" strains isolated from triparental crosses.
For explanation of these results see text and foot-notes on next page.

Table 2 Summary of results.

(a) This column shows only the episomal markers that are relevant. The F-prime factors used were: F8(*gal*$^+$); F42(*lac*$^+$); F13(*tsx*$^+$ *purE*$^+$ *lac*$^+$); F30(*his*$^+$); KLF1(*thr*$^+$ *leu*$^+$) and KLF4(*thr*$^+$ *leu*$^+$ *pro*$^+$).

(b) Samples of 0.1 ml. of the mating mixtures as well as of 10^1 to 10^4 dilutions from triparental matings were plated onto selective agar media containing streptomycin and incubated at 37°C for 48 to 72 hours.

(c) (>) Up to 10 fold difference in number of progeny inheriting the respective markers.
(≫) Greater than tenfold difference.
(=) Approximately similar frequency.

(d) Purified stable "doubles" were used as donors in matings with ED1308(F$^-$ *recA*$^-$) for 30 minutes. Each pair of markers refers to a class of progeny that were obtained.

(e) F42 and F13 were used. The results described refer only to the former since experiments involving F13 did not result in the isolation of any stable "double" strains.

(f) EMB and minimal agar, both media supplemented with either lactose or galactose were used. The results shown in this table refer only to experiments in which minimal lactose and galactose were used. From colonies plated out on EMB galactose no stable "double" strains were isolated and from EMB lactose the frequency of stable "double" strains obtained was 100 times lower than that from minimal lactose. "Doubles" isolated from EMB or minimal media had similar properties.

(g) Changes in acridine orange concentration changed the efficiency of curing but in all curing attempts both *gal*$^+$ and *lac*$^+$ were cured together (see next paragraph).

(h) In later experiments only *gal*$^+$ was transferred and cured by acridine orange; the cured Lac+Gal- derivatives turned out to be females (see text).

(i) Both KLF1 and KLF4 were used. The results were similar in the two cases.

(j) No stable "double" strains were isolated (see text).

(excepting *lac*$^+$), progeny from all matings showed up as standard-size colonies (3mm in diameter) on selective minimal agar after 2-3 days incubation. In contrast those obtained when selection was exerted simultaneously for two markers emerged as very small colonies (only just large enough to be counted) after 6-7 days incubation; by comparison with the colonies obtained in the control crosses these small colonies were neither background nor the product of cross-feeding (or mating on plates). However they could result as a consequence of cross-feeding after mating and segregation have taken place. Cells harbouring either F-prime factor growing in the same colony could reciprocally support their growth to some extent. Also the small size of these colonies may be explained by assuming that because of incompatibility during growth segregation of either episome of a pair occurs and that in an analogous way to abortive transduction the enzymes coded for by the segregated episomes are distributed randomly among the progeny, even if the episome itself is not inherited or if inherited does not replicate, thus allowing a small amount of growth. In such crosses, during the first 48 hours of incubation a few colonies (in the order of 2-3 per plate) showed up as standard size colonies on mating plates onto which samples of the undiluted mating mixtures were plated. Tests involving phenotypic and transfer properties showed that in some cases they represented spontaneous Str^r mutants of one or other of the donor strains; in others they were merodiploids in which one of the episomal markers had become integrated into the host chromosome or revertants of the recipient strain for one or other marker. No detailed studies on such strains were carried out. The absolute number of the progeny when selecting for two markers was 20 to 40% of that obtained when selection was for only one marker. However the absolute yield of stable "double" colonies proved to be similar in both cases.

All clones of the different "doubles" proved to be Rec^- on the basis of their sensitivity to UV irradiation, and when treated with male (MS2) and female (ØII) specific phages all showed the behaviour expected from a typical male strain.

The fact that these "double" strains were Rec^- was not considered sufficient evidence that the "double" phenotype was due to the presence of the two F-prime factors replicating autonomously of each other in the same cell. In fact because of incompatibility this possibility was doubted a priori and recombination of the F-prime factors with each other or with the host chromosome by means of some mechanism other than the Rec system was kept in mind. In order to discriminate between these possibilities two main criteria were adopted. Firstly, spontaneous segregation and curing by acridine orange of either or both markers were considered indicating that the F-prime factors were not integrated into the host chromosome. Secondly, if in crosses of these "double" strains with ED1308 (an F^-Spc^r strain otherwise isogenic with ED1314) the two markers could be transferred independently to different recipient cells, this was taken to suggest that the F-prime factors were independent of the chromosome and of each other for at least part of the time. A general problem using this system is that a pair of F-prime factors that can coexist in one strain may be unable to coexist after both being transferred to another cell. Thus genes carried on a tandem structure of two F-prime factors that was able to exist in one cell but was unstable in another might on the basis of transfer alone be interpreted as a case of two independent structures.

(i) The isolation and characterization of Gal^+Lac^+ strains from F42(lac^+) and F8(gal^+)

Despite the fact that there is not a suitable way to select lac^+ and gal^+ markers simultaneously with the recipient used, a study on cohabitation between

F-prime factors carrying these markers was made since coexistance of F8 and F42 has been claimed (Cuzin and Jacob, 1967b). ED1232 (F*gal*$^+$) and two different F*lac*$^+$ carrying strains, ED1283 (F13) and ED1287 (F42) were used as donors in matings with ED1314. From these matings clones were selected on minimal agar and on EMB, both media being supplemented with either galactose or lactose and containing streptomycin. Gal$^+$ and Lac$^+$ progeny were isolated at about the same frequency, 60% of the female input, after incubation for 48 to 72 hours. The results to be described refer to F42. No stable Gal$^+$Lac$^+$ colonies were isolated on either minimal or EMB media when F13 was used.

On either EMB lactose or galactose, 75% of the Lac$^+$ or Gal$^+$ colonies were sectored; the rest of the colonies, which seemed not to be sectored, were tested by replica-plating on selective media to check whether they also carried the ability to ferment the unselected sugar. Most of them showed the Gal$^+$Lac$^+$ phenotype (replicas were either thick, light or groups of single colonies), but during the purification process either one marker or the other was lost at a very high frequency. Preferential loss of the *lac*$^+$ marker occurs since the number of Gal$^+$Lac$^-$ segregants was ten times higher than that of Gal$^-$Lac$^+$ segregants. However after a fourth purification cycle it was finally possible to isolate from EMB lactose plates rare "stable" colonies approximately 1 out of 200 colonies tested showing the Gal$^+$Lac$^+$ phenotype. The frequency with which these "stable" Gal$^+$Lac$^+$ colonies were isolated was impossible to quantify because of the purification procedure involved. It is possible that the stable "doubles" arose during the purification procedure rather than in the matings themselves.

On minimal lactose plates, the size of the Lac$^+$ colonies was always very small, about 1/5 the size of Gal$^+$ colonies on minimal galactose plates. This

difference in size was also found among progeny from biparental crosses. Unpurified colonies from minimal lactose and galactose plates, like those obtained on EMB plates, were tested for inheritance of the ability to ferment the unselected sugar by replica-plating on minimal agar containing either galactose or lactose. From these 70 - 90% of the colonies selected on minimal lactose plates contained cells that were Gal^+, while only 5% of the colonies selected on minimal galactose plates contained Lac^+ cells. As with those isolated on EMB plates, colonies showing the Gal^+Lac^+ phenotype were purified and despite the high frequency of segregation of Lac^+Gal^- and Gal^+Lac^- colonies (the latter ten times more numerous than the former) it was possible, after several purification cycles, to isolate stable Gal^+Lac^+ clones from both types of minimal media at a frequency 10 times higher than while using EMB plates (Table 2).

Segregation tests carried out on thesestable Lac^+Gal^+ colonies showed a very low rate of segregation (0.5%) for either marker. Fifteen such stable Gal^+Lac^+ colonies, from independent isolates, were treated with acridine orange. This treatment cured these "double" strains of both markers simultaneously, the efficiency of curing depending on the concentration of acridine orange used; concentrations of 10 to 25 µg./ml. had a curing efficiency of 30 to 90%. At all concentrations of acridine orange the two markers were always cured together suggesting that they are carried on a single structure, although it is possible that acridine orange may always cure both episomes together even when in separated structures. These results are therefore different from those obtained from spontaneous segregation, where it was observed that only one marker at a time is segregated. From these results it is possible to conclude tentatively that the two markers remain autonomous from the chromosome, but that even if they are associated with each other for part of the time, they can segregate from each other.

Five purified stable Gal^+Lac^+ isolates were used as donors in crosses with ED1308 (F^- $RecA^-Gal^-Lac^-Spc^r$). After interruption of the mating, 0.1 ml aliquots of a series of dilutions were plated onto minimal and EMB agar each containing spectinomycin. The possibility that the donor culture was a mixed population with cells carrying either one or the other F-prime factor was ruled out by segregation tests of the donor population made at the time the mating was performed. From this mating only two classes of progeny were found (Table 2). Those which had inherited Gal^+ were Lac^- and those inheriting Lac^+ were Gal^-. From this result the simplest conclusion is that in these strains F<u>gal</u>$^+$ and F<u>lac</u>$^+$ can replicate autonomously from each other and from the host chromosome.

Thus the segregation, acridine orange and transfer experiments all demonstrate autonomy of the <u>lac</u>$^+$ and <u>gal</u>$^+$ determinants from the chromosome. However the acridine orange experiment suggest that they are physically linked to each other, although maybe unstably, whereas the segregation and transfer experiments suggest that they are separate structures.

The results from this cross were not reproducible since in matings performed one or two months later using cultures of the same strains, only <u>gal</u>$^+$ was transferred despite the fact that the donors remain Gal^+Lac^+. In these later experiments only <u>gal</u>$^+$ was cured after acridine orange treatment and the cured Lac^+Gal^- derivatives were sensitive to female specific (ØII) and resistant to male specific (MS2) phages, as if <u>lac</u>$^+$ had become integrated into the host chromosome. These results suggest that although in the "double" strains described both <u>gal</u>$^+$ and <u>lac</u>$^+$ were originally both able to replicate autonomously in the same cell, this coexistence was unstable.

The results described suggest that even though there is not an appropriate way of selecting these two markers simultaneously it is possible to isolate colonies carrying both. The incompatibility exerted by the two F-prime factors in this system seems to be asymmetrical in the sense that the F*gal*$^+$ tends to displace F*lac*$^+$; this is suggested by the fact that Gal$^+$Lac$^-$ segregants were found with a much higher frequency than Lac$^+$Gal$^-$ segregants. A similar observation was made by De Haan and Stouthamer (1963).

(ii) *The isolation and characterization of Gal$^+$Leu$^+$ strains from F8(gal$^+$), KLF1(thr$^+$leu$^+$) and KLF4(thr$^+$leu$^+$pro$^+$)*

Triparental crosses using ED1314 as the recipient were performed between ED1232 (F8) in pairwise combinations with ED1291 (KLF1) and ED1294) (KLF4). Here, unlike the case of *gal*$^+$ and *lac*$^+$, the markers transferred in each cross by the two donor F-prime strains can be selected for simultaneously. The absolute number of progeny when selecting for Gal$^+$Leu$^+$ together (40% of the female input) was 20% of that obtained when only Gal$^+$ was selected for. This is due entirely to the poor donorability of the two F*leu*$^+$ strains, since the yield of Leu$^+$ progeny was the same in these triparental crosses as in the biparental control crosses. It must also be mentioned that besides being poor donors, neither KLF1 nor KLF4 inhibit the growth of the female specific phage ØII.

Colonies obtained from 10^3 and 10^4 dilutions of the triparental matings were purified as described previously. Using either KLF1 or KLF4, stable colonies showing the Leu$^+$Gal$^+$ phenotype were isolated from minimal plates, selecting for either one or two markers. From plates selecting for Leu$^+$Gal$^+$ together in crosses involving F8 and KLF1, 2 out of 20 independently isolated

colonies tested were stable Leu^+Gal^+ by the criterion applied, and 5 out of 20 were stable Leu^+Gal^+ when KLF4 was used. When only Gal^+ was selected for, 5 out of 100 colonies using KLF1, and 9 out of 100 using KLF4 were stable Gal^+Leu^+. A similar proportion of stable "doubles" were also obtained when Leu^+ was selected for. In all cases stable Gal^+Leu^+ colonies were obtained after a second purification cycle. These purified "double" colonies were still Rec^- and inhibited the growth of phage ØII. Spontaneous segregation tests showed that only $\underline{gal}^+$ was segregated although at a low frequency (0.5%). However the segregation of Leu^-Gal^+ colonies was not observed either in the presence or the absence of acridine orange; neither was curing observed in control experiments with the parental F-prime strains, ED1291 (KLF1) and ED1294 (KLF4), several thousand colonies of which were tested. KLF1 has previously been reported to be resistant to curing by acridine orange (Palchoudhury and Iyer, 1971b). Therefore although it is possible to conclude that $F\underline{gal}^+$ can replicate autonomously in these "double" strains and that $\underline{leu}^+$ can be separated from $\underline{gal}^+$, it cannot be decided whether in these strains $\underline{leu}^+$ is chromosomal or autonomous.

Purified Leu^+Gal^+ clones were used as donors in matings with ED1308 ($F^-RecA^-Leu^-Gal^-His^-Spc^r$), in which $Leu^+(Spc^r)$, $Gal^+(Spc^r)$ and $Leu^+Gal^+(Spc^r)$ progeny were selected (Table 3). Only two classes of progeny were isolated from these crosses. One class had inherited $\underline{gal}^+$ alone and the other class had inherited $\underline{leu}^+\underline{gal}^+$; no progeny that had inherited $\underline{leu}^+$ alone were obtained. The absence of a class inheriting $\underline{leu}^+$ alone may be a reflection of the poor donorability of both KLF1 and KLF4 and the high efficiency with which $F\underline{gal}^+$ mediate its own transfer, although in spite of this poor donorability some Leu^+Gal^- progeny would have been expected on the basis of the number of progeny tested, if the transfer of $\underline{leu}^+$ was completely independent of that of $\underline{gal}^+$ (see Table 3). Other models will be discussed later.

Donor phenotype	Markers selected	Number of colonies tested	colonies inheriting leu^+	colonies inheriting gal^+	colonies inheriting leu^+ gal^+
Gal^+Leu^+ (KLF1)	leu^+	500	500	500	500
" " "	gal^+	100	19	100	19
" " "	leu^+ gal^+	100	100	100	100
" " (KLF4)	leu^+	400	400	400	400
" " "	gal^+	100	50	100	50
" " "	leu^+ gal^+	100	100	100	100

Table 3. Coinheritance of episomal markers from matings using donors with "double" phenotype.

Exponential cultures in L-broth of each of the stable "doubles" carrying episomal markers from two different F-prime factors, and of ED1308 each containing approximately 2×10^8 cells./ml. were crossed 1:5 at 37°C for 30 minutes. After interruption of the matings, 0.1 ml of 10^1 to 10^4 dilutions of each mating were plated onto spectinomycin minimal agar selective for each marker and both markers simultaneously and incubated at 37°C for 5 days. From 100 to 500 single well separated colonies from each class of minimal plates were patched on minimal agar of the same composition and then replica-plated on selective plates to check coinheritance.

The results, which are summarized in Table 2, show as do those with the Flac$^+$ and Fgal$^+$, the isolation of strains which have inherited episomal markers from two different F-prime strains. Of these gal$^+$ has been shown to be harboured in an autonomous state by segregation tests, acridine orange treatment, and the criterion of transfer. In contrast, it was impossible to show whether the leu$^+$ marker (carried either by KLF1 or by KLF4) was autonomous or integrated, or whether it was or was not associated with gal$^+$. On the one hand, leu$^+$ can be transferred, but only into recipients which also receive gal$^+$ suggesting that these genes are associated, although the absence of Leu$^-$Gal$^-$ segregants suggests that leu$^+$ is not associated with gal$^+$. On the other hand, not all Gal$^+$ progeny have received leu$^+$ (or else can stably cohabit with it in the new recipient).

(iii) The isolation and characterization of His$^+$Leu$^+$ strains from F30(his$^+$), KLF1(thr$^+$leu$^+$) and KLF4(thr$^+$leu$^+$pro$^+$)

Triparental crosses using ED1314 as the recipient were performed between ED1229 (F30) in pairwise combinations with ED1291 (KLF1) and ED1294 (KLF4). The number of progeny obtained when selecting for his$^+$leu$^+$ together was reduced to about 25% of the absolute number of progeny when selecting for his$^+$ alone (65% of the female input) (see Table 2). In contrast, the yield of progeny obtained when selecting for leu$^+$ alone was the same as that obtained when selecting for leu$^+$his$^+$ together. This again can be attributed to the poor donorability of both KLF1 and KLF4.

When KLF4 was used as one of the donors, the proportion of stable Leu$^+$His$^+$ "doubles" that were isolated was about twice of that when KLF1 was used. These stable Leu$^+$His$^+$ colonies were obtained after a second cycle of

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purification. The frequencies with which they were isolated were similar to those described previously when F*gal*$^+$ was used instead of F*his*$^+$. These purified stable Leu$^+$His$^+$ colonies were Rec$^-$ and inhibited the growth of phage ØII. From these "doubles", *leu*$^+$ was never lost, neither spontaneously nor in the presence of acridine orange. Only *his*$^+$ was segregated although at a very low frequency (0.5%), which suggests that F*his*$^+$ replicates autonomously in their "doubles". The absence of Leu$^-$His$^-$ segregants seems to suggest that *leu*$^+$ is not associated with *his*$^+$. However neither an episomal nor a chromosomal state of *leu*$^+$ can be decided on the basis of this information alone.

Purified Leu$^+$His$^+$ clones were crossed with ED1308 (F$^-$ RecA$^-$Leu$^-$Gal$^-$His$^-$Spc^r) and Leu$^+$ (Spc^r), His$^+$(Spc^r) and Leu$^+$His$^+$(Spc^r) progeny were selected from such crosses (Table 4). Only two classes of progeny were obtained from such matings. One class had inherited *his*$^+$ alone and the other class had inherited *leu*$^+$*his*$^+$; no progeny which had inherited *leu*$^+$ alone was observed. Again it is very difficult to give a clear explanation of the nature of these "doubles". The absence of progeny inheriting *leu*$^+$ cannot be satisfactory explained on the basis of the poor donorability of both KLF1 and KLF4 alone. These results are very similar to those obtained in the previous section where F*gal*$^+$ was used instead of F*his*$^+$. The segregation and transfer experiments suggest that *his*$^+$ is harboured in an autonomous state. In contrast, these results do not help to distinguish whether the *leu*$^+$ marker (carried either by KLF1 or KLF4) was autonomous or integrated, or whether or not it was associated with *his*$^+$.

(iv) The isolation and characterization of Lac$^+$His$^+$ strains from F42(*lac*$^+$) and F30 (*his*$^+$)

Stable Lac$^+$His$^+$ strains which seem to be carrying episomal markers from both donors were isolated from triparental crosses using ED1287 (F42) and ED1229 (F30) as the donor strains and ED1314 as the recipient strain (see

Donor phenotype	Markers selected	Number of colonies tested	colonies leu^+	colonies his^+	colonies inheriting leu^+ his^+
His^+ Leu^+ (KLF1)	leu^+	300	300	300	300
" " "	his^+	100	12	100	12
" " "	leu^+ his^+	100	100	100	100
" " (KLF4)	leu^+	400	400	400	400
" " "	his^+	100	41	100	41
" " "	leu^+ his^+	100	100	100	100

Table 4. Coinheritance of episomal markers from matings using donors with "double" phenotype.

Exponential cultures in L-broth of each of the stable "doubles" carrying episomal markers from two different F-prime factors, and of ED1308 each containing approximately 2×10^8 cells./ml. were crossed 1:5 at 37°C for 30 minutes. After interruption of the matings, 0.1 ml of 10^1 to 10^4 dilutions of each mating were plated onto spectinomycin minimal agar selective for each marker and both markers simultaneously and incubated at 37°C for 5 days. From 100 to 400 single well separated colonies from each class of minimal plates were patched on minimal agar of the same composition and then replica-plated on selective plates to check coinheritance.

Table 2). Colonies obtained from 10^3 and 10^4 dilutions of the triparental mating were purified as described previously. From minimal plates selecting for lac$^+$ his$^+$ together, 1 out of 60 independently isolated colonies tested was stably Lac$^+$His$^+$ by the criterion applied in this thesis. When selection was for either lac$^+$ or his$^+$, about 1% of the colonies tested were stable Lac$^+$His$^+$. Three purification cycles were required to obtain these stable "doubles". During the purification process, preferential loss of the lac$^+$ marker was suggested since the yield of Lac$^-$His$^+$ segregants was ten times higher than that of Lac$^+$His$^-$ segregants.

Segregation tests with single purified stable colonies showed that only Lac$^+$His$^-$ colonies were segregated at a frequency between 0.5 to 1%. Treatment with acridine orange neither increased this loss nor cured the Lac$^+$His$^+$ strains of the lac$^+$ marker. In crosses between His$^+$Lac$^+$ strains and ED1308 (Table 5), Lac$^+$His$^+$ and His$^+$ progeny, but not Lac$^+$ progeny were produced. These results are those analogous to those observed in (ii) and (iii) with respect to the transfer behaviour of the "double" strain and the pattern of spontaneous segregation and curing by acridine orange; here Lac$^+$ is similar to Leu$^+$. As in the previous cases the nature of these "doubles" is unclear. The possibility of dealing with Lac$^+$ revertants can be excluded since (a) the frequency with which His$^+$Lac$^+$ colonies were isolated was too high to be due to reversion, and (b) both lac$^+$ and his$^+$ are transferred (together) to ED1308.

(v) The failure to isolate stable Lac$^+$Leu$^+$ and Gal$^+$His$^+$ strains from F42(lac$^+$), KLF1(thr$^+$leu$^+$), KLF4(thr$^+$leu$^+$pro$^+$) and from F8(gal$^+$), F30(his$^+$) respectively

Using the same strains (in different combinations) and the same techniques as in the previous cases, no stable clones carrying either of these two pairs of episomal markers were isolated. Regarding the pairs F42, KLF1 and F42,

Donor phenotype	Markers selected	Number of colonies tested	colonies inheriting lac^+	his^+	$lac^+ his^+$
$Lac^+ His^+$	lac^+	300	300	300	300
" "	his^+	100	8	100	8
" "	$lac^+ his^+$	100	100	100	100

Table 5. Coinheritance of episomal markers from matings using donors with "double" phenotype.
Exponential cultures in L-broth of each of the stable "doubles" carrying episomal markers from two different F-prime factors, and of ED1308 each containing approximately 2×10^8 cells./ml. were crossed 1:5 at 37°C for 30 minutes. After interruption of the matings, 0.1 ml of 10^1 to 10^4 dilutions were plated onto sepctinomycin minimal agar selective for each marker and both markers simultaneously and incubated at 37°C for 5 days. From 100 to 300 single well separated colonies from each class of minimal plates were patched on minimal agar of the same composition and then replica-plated on selective plates to check coinheritance.

KLF4, the poor donorability of Fleu$^+$ strains and the very poor growth that was observed on the selective minimal lactose agar make it possible that the failure to isolate Leu$^+$Lac$^+$ strains was due merely to technical reasons.

Regarding the pair F8 and F30, the usual high number of standard-size colonies were found on minimal agar selecting for either gal$^+$ or his$^+$. None of several hundred of these colonies tested showed that Gal$^+$His$^+$ phenotype; therefore these colonies have either inherited only one F-prime factor or if they have inherited both one or other of them was rapidly segregated out. On minimal plates selecting for both markers together an appreciable number of very small colonies, not detected by cross-feeding tests, were obtained after 5 days of incubation; in addition, a few standard-size colonies arose on plates spread with the undiluted mating mixture. From these standard-size colonies, only gal$^+$ was transferred to the recA recipient ED1308. These colonies therefore seem to represent recipient cells that are recombinants or revertants for the His$^+$ phenotype and carry Fgal$^+$ autonomously. They were fairly stable and only the Gal$^+$ phenotype was cured after acridine orange treatment. The small colonies, on the other hand, were extremely unstable and on purification only Gal$^+$His$^-$ or Gal$^-$His$^+$ clones were obtained. These clones were of standard-size, and segregated and transferred gal$^+$ and his$^+$ respectively at the frequencies observed in biparental control crosses with the parent F-prime strains.

The fact that both the Fgal$^+$ and Fhis$^+$ carrying strains grow very well, mediate their own transfer with high efficiency and show this high degree of incompatibility towards each other makes them suitable for the further studies that will be described in the next section.

D. Discussion

The results of this experimental section are summarized in Table 2. As can be seen most triparental crosses gave stable clones carrying episomal markers from each of the two parental F-prime factors. The exception were the pairs in which F*leu*$^+$, F*lac*$^+$ and F*gal*$^+$, F*his*$^+$ were used, and those involving F13. The frequency of these stable "doubles" depended on the selection used; thus on minimal agar when selection was for only one donor episomal marker, this frequency, relative to the absolute number of progeny for that particular marker, was ten times lower than the frequency when both donor episomal markers were selected for together.

Three possible explanations can be given to account for the presence of both episomal markers in the same cell: (a) the two F-prime factors are coexisting stably and replicating independently of each other and of the host chromosome, (b) they are physically joined in a hybrid episomal structure, which may be unstable, and (c) one of the episomal markers has become integrated in the host chromosome by a mechanism other than recombination by the host *recA* promoted recombination system. This integration could be unstable and the integrated F-prime factor might alternate between the integrated and the autonomous state.

The stable "doubles" isolated in this section can be grouped in two categories according to their segregation, curing and transfer behaviour; one group comprises the Lac$^+$Gal$^+$ "double" strains, and the other group comprises His$^+$Leu$^+$, Gal$^+$Leu$^+$ and His$^+$Lac$^+$ "double" strains.

gal$^+$ and *lac*$^+$ seem both to be autonomous in the Gal$^+$Lac$^+$ strains that were isolated. The evidence that they are present as stably coexisting F-prime factors unaffected by incompatibility (model a) is two fold. First,

spontaneous independent segregation of gal$^+$ and lac$^+$ is observed although at only a very low frequency and second, gal$^+$ and lac$^+$ are transferred separately to a further recA recipient. However the fact that acridine orange treatment of these Gal$^+$Lac$^+$ strains always cured both markers together may suggest that these markers are physically joined (model b). However it is possible that both markers may be always cured together even when existing in separate structures. A possible explanation for these results is that an equilibrium exist between a gal$^+$lac$^+$ structure and separate gal$^+$ and lac$^+$ structures, and that it is these latter structures that are transferred and segregated. Another explanation for the independent transfer (but not for the independent segregation) is that gal$^+$ and lac$^+$ are transferred on one structure, which is then broken down in the recipient strain. It was subsequently concluded that whatever the state in which lac$^+$ existed, this state was unstable since in later experiments only gal$^+$ could be cured or transferred. The lac$^+$ marker had apparently recombined into the host chromosome since cured Lac$^+$Gal$^-$ derivatives were sensitive to female specific (ØII) phage.

Both the leu$^+$ F-prime factors, KLF1 and KLF4, gave rise to stable Leu$^+$Gal$^+$ and Leu$^+$His$^+$ "doubles". From such stable clones only gal$^+$ and his$^+$ markers were spontaneously segregated and cured by acridine orange: in crosses with a recA recipient, Leu$^+$ progeny were invariably Gal$^+$ or His$^+$, although Gal$^+$Leu$^-$ and His$^+$Leu$^-$ progeny could be isolated. An analogous pattern was observed with clones showing the His$^+$Lac$^+$ phenotype. Thus only his$^+$ was spontaneously segregated and cured by acridine orange; it could also be observed that on transfer only His$^+$Lac$^+$ and His$^+$Lac$^-$ progeny but not His$^-$Lac$^+$ progeny were found.

The results described are difficult to explain by any of the models proposed. In each case acridine orange treatment only cured either gal$^+$ or his$^+$ and has no curing effect on either leu$^+$ or lac$^+$; this suggested that

both episomal markers (or F-prime factors) in each pair exist as two separate structures, at least part of the time. The possibility that any of them had become integrated into the chromosome (as suggested by model c) is less probable since the two markers involved in each pair are transferred to a further recA recipient, although the possibility that Fleu$^+$ and Flac$^+$ alternate between an integrated and an autonomous state cannot be ruled out.

One hypothesis which would explain why in matings in which either Leu$^+$Gal$^+$, Leu$^+$His$^+$ or His$^+$Lac$^+$ "doubles" were used as donors, leu$^+$ and lac$^+$ are not inherited alone is to assume that in the "double" strains both Fleu$^+$ and Flac$^+$ are defective in their replication. These mutants could be autonomous from Fgal$^+$ and Fhis$^+$ and would be able to replicate themselves by using the replication enzymes made by either of these wild type F-prime factors. These defective Fleu$^+$ and Flac$^+$ could be transferred alone to the new recipient where failing to replicate would be diluted out during growth. In this way their transfer, under the conditions used, would be undetected.

An alternative hypothesis that may explain the apparent absence of a class of progeny inheriting leu$^+$ of lac$^+$ alone would be to assume that in fact the pairs of markers leu$^+$gal$^+$, leu$^+$his$^+$ and his$^+$lac$^+$ are always transferred together and that in the new recipient incompatibility between members of each pair breaks down this association resulting in either leu$^+$ or lac$^+$ being segregated out in all cases. In this case what seems to be a case of independent transfer of either gal$^+$ or his$^+$ alone would be nothing more than the result of the expression of incompatibility which in these systems seems to be exerted to a greater extent by both Fgal$^+$ and Fhis$^+$ than by Fleu$^+$ and Flac$^+$. A mating between these "doubles" and an F$^-$recA$^+$ recipient might have helped to test this hypothesis since the segregated marker might have been rescued by recombination with the chromosome.

The principal conclusion from this group of experiments is that in no case has a strain been isolated which fulfils even the simplest criteria for cohabitation of two independent F-prime factors. However, the nature of the strains with the "double" phenotype that were isolated is of interest. Since they arose in cells which retain the RecA$^-$ phenotype, it is possible to conclude that their genesis does not involve the RecA function. Since at least in the newly isolated "doubles" each marker can be transferred to a further recA strain, it seems likely that they are all carried autonomously. It therefore appears that in each case, and at least during part of the time, the two F-prime factors involved in each pair may have been able to coexist.

Of the several F-prime factors used in this study KLF1 and KLF4, in contrast with F*gal*$^+$, F*lac*$^+$ and F*his*$^+$, are odd in the sense that they do not inhibit the multiplication of the female specific phage ØII; this atypical F-prime behaviour is most evident in KLF4. The relationship between KLF4 and other F-prime factors will be further examined in section III. Further studies on cohabitation involving mutagenesis of F8 and F30 carrying strains, and ED1314 are described in the next section.

SECTION II

ISOLATION OF A MUTANT STRAIN ALLOWING COHABITATION OF TWO OR THREE F-PRIME FACTORS

A. Introduction

Attempts to find cases of cohabitation between different non-mutagenized F-prime factors have been described in section I, where it is shown that none of the stable "double" strains isolated appeared to be simple cases of cohabitation of the F-prime factors involved. This section is concerned with the mutagenesis of each of a pair of F-prime factors and of the recipient strain in order to select mutants that allow cohabitation. On the basis of the experiments described in section I, the F-prime factors selected were F8(*gal*$^+$) and F30(*his*$^+$), because (a) they allowed good growth and mediate their own transfer with high efficiency, and (b) they failed to give Gal$^+$His$^+$ "doubles".

B. Mutagenesis of the F-prime strains

(i) Techniques

(a) Biparental crosses: NTG-mutagenized exponential cultures (see Materials and Methods) of each of the F-prime strains ED1229 (F30) and ED1232 (F8) were crossed 1:10 in independent matings with ED1314. After 30 minutes the matings were interrupted, and the cultures were centrifuged, washed and resuspended in liquid minimal media selective for either His$^+$ or Lac$^+$ and supplemented with streptomycin, and were incubated at 37°C for several hours. Samples of 0.1 ml. were then diluted into 10 ml. of L-broth and incubated

until late stationary phase in order to obtain populations in the F^- phenocopy state. These were then used as recipients in crosses with the other (non-mutagenized) exponentially grown F-prime strain using a 1:10 ratio of donor to recipient. After mating for 30 minutes at 37°C the matings were interrupted. Aliquots of 10^0 to 10^4 dilutions were plated onto streptomycin-supplemented selective minimal agar on which only the offspring which had inherited episomal markers from both F-prime strains could grow. These plates were incubated at 37°C for 6 days. This extended period of incubation was required for small colonies to appear. Purification of the resulting colonies was carried out as described in Section I.

(b) Triparental crosses: exponentially growing populations of both mutagenized F-prime strains were mated in the proportions 5:5:1 with ED1314 in a triparental cross. After interruption of the mating, plating and the selection and purification of progeny were performed as above.

(ii) Results

The results from both experiments are shown in Table 6. In each case colonies of two different sizes showing the Gal^+His^+ phenotype were obtained. On plates onto which aliquots of the 10^0 to 10^2 dilutions were plated, standard-size colonies started to show up after 48 hours of incubation; on the basis of the absolute yield of progeny obtained, these were ten times more numerous in biparental than in triparental crosses. On those plates on which samples of 10^3 and 10^4 dilutions were spread, only small colonies had arisen after 6 days of incubation; these small colonies were more numerous in triparental than in biparental crosses. Colonies of both sizes and from both biparental and triparental crosses were purified and their phenotypes checked as described in section I. Colonies having the same size isolated from both biparental

Crosses	Colonies inheriting[a] gal$^+$ his$^+$ Standard-size colonies ($\times 10^{-3}$)	Small[b] colonies ($\times 1$)
(i) biparental		
ED1229(NTG) xED1314xED1232	6.3	40
ED1232(NTG) xED1314xED1229	4.5	32
(ii) triparental		
ED1229(NTG)xED1232(NTG)xED1314	0.3	60

Table 6. Matings using NTG-mutagenized F-prime strains.

(i) Exponential L-broth cultures of each of the NTG-mutagenized F-prime strains and of the non-mutagenized F$^-$ strain were mated 1:10 in single crosses, washed and resuspended in selective liquid minimal medium containing streptomycin and allowed to grow for several hours. Aliquots of 0.1 ml., were then diluted in L-broth and further incubated until late stationary phase. These cells in F$^-$ phenocopy state were used as recipients in 1:10 crosses with the other (non-mutagenized) F'-strain.

(ii) Exponential cultures of both mutagenized F-prime strains were simultaneously mated 5:5:1 in triparental cross with ED1314.

Matings in (i) and (ii) were allowed to proceed for 30 minutes at 37°C before interruption. The number of donor and recipient cells was about 2 x 10^8 cells/ml. Samples of 0.1 ml of 10^0 to 10^4 dilutions were spread on selective minimal agar containing streptomycin. Plates were incubated for 6 days.

(a) Colonies per 100 cells of the limiting parent.

(b) The size of the small colonies was 1/5 that of the standard-size colonies.

and triparental crosses showed the same behaviour. Both small and standard-size colonies were Rec^-, as shown by their high degree of sensitivity to UV irradiation.

The small colonies showed a high frequency of segregation of both Gal^- and His^- clones and after a second cycle of purification all colonies were either Gal^+His^- or Gal^-His^+. These small colonies did not seem to be any different from those described in section I for the same pair of F-prime factors, and seem to represent recipient cells which have received both F-prime factors but are unable to maintain F8 and F30 in the same cell.

Only 10% of the standard-size colonies obtained from either biparental or triparental crosses segregated either marker at high frequency; the remainder were stable Gal^+His^+ clones in which only the $\underline{gal}^+$ marker was segregated out, though at a very low frequency (0.8%). This loss was not increased after treatment with acridine orange. When 200 independently isolated colonies of such stable Gal^+His^+ colonies were crossed by replica-plating with ED1308 (F^- $RecA^-Gal^-His^-Spc^r$) only $\underline{gal}^+$ was transferred.

These results suggest that in these stable Gal^+His^+ clones, $\underline{gal}^+$ is replicated autonomously. In contrast, the $\underline{his}^+$ marker seems to have become integrated into the chromosome, since no His^- segregants were observed and since $\underline{his}^+$ was not transferred either alone or with $\underline{gal}^+$. These standard-size colonies therefore seem to be similar to those described in section I for the same pair of F-prime factors. However the frequency with which these were isolated was much higher than that observed either from spontaneous reversion of the recipient population alone or by spontaneous integration of the $\underline{his}^+$ marker in the chromosome (see section I).

These results suggest that mutagenesis of the F-prime strains under the comditions used did not result in the formation of F-prime factors able to

form stable "double" strains. In all of 200 standard-size Gal$^+$His$^+$ colonies tested, his$^+$ seems always to be integrated while gal$^+$ was autonomous. Why none of the colonies tested showed gal$^+$ in the integrated state and his$^+$ in the autonomous state is not clear.

C. Mutagenesis of the F$^-$ strain

(i) Results

ED1314 was treated with nitrosoguanidine as described in Materials and Methods. An exponential mutagenized culture of this strain was mated in a triparental cross with exponential cultures of ED1232(F8) and ED1229(F30) used as donors. The proportion of males to female was 5:5:1. The mating procedure and the selection of progeny were similar to those in the triparental cross just described.

The results of this mating are shown in Table 7. Again two classes of colony-sizes were obtained. However on this occasion the number of standard size colonies was 300 fold higher than in the previous matings, while the number of small colonies was reduced to 1/3. The segregation patterns with both types of colonies were similar to those described in the previous experiments. After a second cycle of purification small colonies became either Gal$^+$His$^-$ or Gal$^-$His$^+$ while the standard-size colonies remained Gal$^+$His$^+$.

These stable Gal$^+$His$^+$ standard-size colonies could be grouped into two classes according to their segregation patterns. In one class, only segregation (0.5%) of the gal$^+$ marker was observed: these colonies therefore seem to be similar to those obtained in the previous experiments in which the F-prime strains were mutagenized and to those in section I. In the second class, segregation of either marker occurred also at a low frequency (0.5%).

Cross	Colonies inheriting *gal*$^+$ *his*$^+$(a): Standard-size colonies	Small(b) colonies
ED1232 x ED1229 x ED1314(NTG)	0.1	16

Table 7. Triparental matings with NTG-mutagenized F$^-$ strain. Exponential cultures in L-broth of both F-prime strains and the mutagenized F$^-$ strain, each at about 2 x 10^8 cells/ml, were mated 5:5:1 at 37°C for 30 minutes. After interruption aliquots of 0.1 ml of 10^0 to 10^4 dilutions of the mating mixture were plated onto selective minimal agar containing streptomycin and incubated for 6 days.

(a) Colonies per 100 recipient cells.

(b) The size of the small colonies was 1/5 that of the standard size colonies.

Treatments with acridine orange did not increase this loss, although simultaneous loss of both markers was observed in a few cases. These colonies were still Rec^- and were also sensitive to phage MS2 and resistant to phage ØII. These observations suggest that colonies belonging to this last class seem to harbour both F-prime factors autonomously.

Fifteen purified standard size Gal^+His^+ colonies in which segregation of both markers was observed were next tested for their ability to transfer these markers in 1:5 matings with ED1308 (F^- recA his gal spc). The results are shown in Table 8. Only ED2602, ED2604, ED2618 and ED2620 transferred both markers at a reasonably high frequency, although this frequency was not as high as that given by the single F-prime strains used in control experiments. The rest of the donor Gal^+His^+ strains tested transferred gal^+, but not his^+. It is possible therefore that although they had originally carried gal^+ and his^+ autonomously, the his^+ marker had become integrated into the chromosome.

From matings in which ED2602, ED2604, ED2618 and ED2620 were used as donors, standard-size colonies started to show up on minimal plates selecting for either gal^+ or his^+ after 2 days incubation; it was found that those which had inherited gal^+ were all his^- while those inheriting his^+ were all gal^-; none of several hundred colonies tested had inherited $gal^+ his^+$ together. However on minimal plates selecting for $gal^+ his^+$ together, a considerable number of small colonies started to show up after 4 days incubation. These small colonies represented recipient cells which had inherited both markers simultaneously; on restreaking they segregated standard-sized Gal^+His^- and Gal^-His^+ clones. This observation shows that transfer of both markers to a common recipient occurs, but because of incompatibility, one or other of them is then segregated out.

Donor	Markers selected (a)		
	his$^+$	*gal*$^+$	*his*$^+$ *gal*$^+$ (b)
ED2601	10^{-4}	17	10^{-5}
ED2602	16	21	10^2
ED2604	23	30	10^2
ED2605	10^{-4}	22	10^{-5}
ED2606	10^{-4}	43	10^{-5}
ED2607	10^{-4}	32	10^{-5}
ED2608	10^{-4}	29	10^{-5}
ED2610	10^{-4}	25	10^{-5}
ED2611	10^{-4}	34	10^{-5}
ED2612	10^{-4}	39	10^{-5}
ED2614	10^{-4}	18	10^{-5}
ED2615	10^{-4}	30	10^{-5}
ED2616	10^{-4}	26	10^{-5}
ED2618	27	31	10^2
ED2620	20	30	10^2
ED1320 (F*gal*$^+$)	10-5	67	-
ED1323 (F*his*$^+$)	53	10-5	-

Table 8. Donorability of His$^+$Gal$^+$ colonies.
Exponential cultures in L-broth of each of the donors and of ED1308 (F$^-$ *recA gal his spc*) as well, each at about 2×10^8 cells/ml., were mated 1:5 at 37°C for 30 minutes. After interruption 0.1 ml from 10^{-1} to 10^{-4} dilutions were plated on selective minimal agar containing spectinomycin and incubated at 37°C for 5 days. Viable counts of the donors (excepting ED1320 and ED1323) were made on minimal agar selective for Gal$^+$His$^+$ and containing streptomycin.

(a) Colonies per 100 donor cells.

(b) These colonies were very small and showed up after 4 days incubation.

A point of interest was that colonies from ED2602, ED2604, ED2618 and ED2620, when grown on minimal or nutrient agar are mucoid and tend to grow very large if incubated or kept at room temperature for more than 48 hours. When smears from these mucoid colonies were observed in the light microscope the bacilli did not look any different from ordinary E.coli.

We conclude that strains ED2602, ED2604, ED2618 and ED2620 carry his^+ and gal^+ in separate $Fhis^+$ and $Fgal^+$ structures. Since both phenotypes, Gal^+His^- and Gal^-His^+, are segregated at a low frequency and both $Fhis^+$ and $Fgal^+$ are transferred independently, it is possible that they indeed exist as cohabiting F-prime factors. The fact that his^+ and gal^+ cannot persist together stably in strain ED1308 or in ED1314 indicates that his cohabitation is a function of the host cells rather than the F-prime factors themselves.

D. Reconstitution of strains harbouring both gal^+ and his^+

Since the cohabitation just described seems to depend upon the host cells rather than on the F-prime factors, an experiment was performed to establish whether or not derivatives of one of these strains, ED2620, in which both episomes had been cured could be used to reconstitute the "double" phenotype. In spite of the low efficiency of curing by acridine orange, three different derivatives were isolated after acridine orange treatment; ED2713 (His^+Gal^-), ED2714 (Gal^+His^-) and ED2745 (His^-Gal^-). Both ED2713 and ED2714 were typical F-prime strains in the sense that they were sensitive to phage MS2 and resistant to phage ØII, and in that each of them transferred its F-prime factor to a further recA recipient at a high frequency. ED2745 behaved as a typical F^- strain, being resistant to phage MS2 and sensitive to phage ØII

and being a good recipient in crosses with F-prime-carrying donor strains. ED2713, ED2714 and ED2745 all retained the mucoid phenotype characteristic of ED2620.

Exponential cultures of ED1229(F30) and ED1232(F8) were mated 1:5 in single crosses with ED2745 for 30 minutes. After interruption samples of the mating mixtures (cultures A and B respectively) were grown in liquid minimal media selective for each particular episomal marker and incubated for 6 to 8 hours. Streptomycin was used to kill the donors. Samples of 0.1 ml. were then diluted in 10 ml. of L-broth and incubated until late stationary phase. These F^- phenocopies as well as F^- phenocopies of ED2713 and ED2714 (see above) were used as recipients in 1:5 single crosses in which ED1229 and ED1232 were the donors. Matings were allowed to proceed for 30 minutes, and after interruption, dilutions of the matings mixtures were plated onto minimal agar selective for Gal^+His^+ containing streptomycin. The results of these matings and the strain numbers given to the newly formed Gal^+His^+ "doubles" strains are shown in Table 9. Only standard size colonies at the expected high frequency were obtained.

Colonies from these matings were purified and tested for segregation. In all cases segregation of either marker was low (0.06%) and again treatment with acridine orange did not increase this loss; however curing of both markers was observed in a few cases. These results suggest that both markers are autonomous at least part of the time. In order to confirm the presence of both F-prime factors in these Gal^+His^+ strains, they were further crossed with ED1308. The results of matings with representative Gal^+His^+ strains are also shown in Table 9. All these newly made Gal^+His^+ "double" strains were mucoid like ED2620 and retained the ability to transfer both markers independently to the new recipient. This mucoid phenotype was never transferred by either of the F-prime factors. Standard-size colonies showing

(1)	(2)	(3)	(4)		(5)	
Donor	Recipient F^- phenocopies	Markers selected (a) *gal*$^+$ *his*$^+$	Representative Gal^+His^+ progeny	recipient	Marker selected (a) *gal*$^+$	*his*$^+$
ED1232 (F*gal*$^+$)	ED2713 (F*his*$^+$)	24	ED2715	ED1308	12	26
ED1232 (F*gal*$^+$)	Culture A(F*his*$^+$)	37	ED2767	ED1308	6	21
ED1229 (F*his*$^+$)	ED2714 (F*gal*$^+$)	45	ED2716	ED1308	5	16
ED1229 (F*his*$^+$)	Culture B(F*gal*$^+$)	43	ED2775	ED1308	7	22

Table 9. Reconstitution and donorability of stable Gal^+His^+ "double" strains. Exponential cultures of ED1232 and ED1229 and late stationary cultures of ED2713, ED2714 and Cultures A and B (see text) were mated 1:5 in single crosses for 30 minutes (columns 1 and 2). After interruption 0.1 ml of 10^1 to 10^4 dilutions were plated onto selective minimal agar containing streptomycin. Plates were incubated for 37°C for 4 days. The results of these matings are shown in column 3. From these, single purified colonies grown to exponential phase (numbered in column 4) were used as donors in further 1:5 matings with ED1308 for 30 minutes. Samples of 0.1 ml of 10^1 to 10^4 dilutions were spread onto selective minimal agar containing spectinomycin. Plates were incubated at 37°C for 4 days. The results of these matings are shown in column 5. Donors and recipients were at about 2×10^8 cells/ml.

(a) Colonies per 100 donor cells.

inheritance of either gal$^+$ or his$^+$ emerged on the selective minimal plates after the first 48 hours of incubation. These results suggest that in these Gal$^+$His$^+$ "double" strains, as in ED2620, gal$^+$ and his$^+$ are carried in a form that can give rise to separate Fgal$^+$ and Fhis$^+$ elements. They support the hypothesis that the autonomous cohabitation of his$^+$ and gal$^+$ in the same cell, in whatever manner, is due to a property of the host cell rather than of the F-prime factors.

E. Cohabitation of F8(gal$^+$), F30(his$^+$) and KLF4(thr$^+$leu$^+$pro$^+$)

In order to see whether a further F-prime factor could coexist in strains ED2602 and ED2620 in the presence of both the Gal$^+$ and the His$^+$ phenotype, single matings between exponential cultures of ED1294(KLF4) as donor and F$^-$ phenocopies of strains ED2602 and ED2620 as recipients were carried out. The proportion of donors to recipient cells was 1:5. After 30 minutes the matings were interrupted and dilutions of the mating mixtures were plated onto streptomycin-supplemented selective minimal agar on which different classes of clones inheriting Leu$^+$ could be selected. The results of these matings are presented in Table 10. In all plates mucoid colonies of different sizes arose after 4 days of incubation. This prolonged period of incubation was necessary to allow smaller colonies to reach a standard-size.

Although inheritance of leu$^+$ was observed in all minimal plates on which this marker was selected, the efficiency of leu$^+$ inheritance was dependent upon the selection used. Thus on plates on which Leu$^+$Gal$^+$His$^+$ progeny were selected, the inheritance of leu$^+$ was much lower than when leu$^+$ was simultaneously selected with only gal$^+$ or his$^+$ or when it was selected alone. It can also be seen that leu$^+$ displaces either his$^+$ or gal$^+$ in the recipient. These results showed that under the conditions used, it was possible to

Cross	Markers selected (a)				
	leu$^+$	leu$^+$his$^+$	gal$^+$his$^+$	leu$^+$gal$^+$	leu$^+$gal$^+$his$^+$
ED1294(KLF4) x ED2602	0.64	0.25	37	0.70	0.01
ED1294(KLF4) x ED2620	0.85	0.20	34	0.58	0.01

Table 10. Introduction of a third F'-factor into Gal$^+$His$^+$ "double" strains.

Exponential cultures of ED1294 were crossed 1:5 in single matings with F$^-$ phenocopies of ED2602 and ED2620 for 30 minutes. Samples of 0.1 ml of 10^1 to 10^4 dilutions were plated on selective minimal agar containing streptomycin, and the plates were incubated at 37°C for 4 days. Donor and recipients were at about 2 x 10^8 cells/ml.

(a) Colonies per 100 donor cells.

isolate colonies which show the Leu$^+$Gal$^+$His$^+$ phenotype suggesting a possible inheritance of KLF4. These Leu$^+$Gal$^+$His$^+$ were Rec$^-$ on the basis of their sensitivity to UV irradiation.

To test for coinheritance, single unpurified but well separated standard-size colonies picked from each type of selective plate, onto which 10^4 dilutions of the mating mixtures had been spread, were patched on non-selective minimal agar and, after growth, were replica-plated onto selective minimal plates on which the presence of each of the three episomal markers could be detected. Streptomycin was used in all plates. The results of this test are shown in Table 11. As can be seen, colonies showing the Gal$^+$His$^+$Leu$^+$ phenotype were found among all classes tested with a frequency which depended on the particular class tested. Also it can be observed that leu$^+$ displaced either gal$^+$ or his$^+$ but not both; the displacement of either marker also varied according to the particular class tested. However it must be pointed out that in this test only standard-size colonies were used; perhaps if small colonies or a larger number of standard-size colonies had been tested a complete range of segregation classes might have been found.

Purified Leu$^+$Gal$^+$His$^+$ clones were then tested for segregation, curing and transfer of the different markers. Each of the three markers was segregated at a low frequency (0.2 - 0.9%), which was not increased by growth in the presence of acridine orange. In no case was simultaneous loss of all three markers observed, although in a few cases simultaneous loss of two markers did occur. When these Leu$^+$Gal$^+$His$^+$ clones were crossed with ED1308, after 4 days of incubation different sizes of colonies showed up on separate selective minimal plates for each of the markers. Among several hundred standard-size colonies tested only two classes of progeny were found; these had inherited either gal$^+$ or his$^+$. Leu$^+$ did not seem to be inherited either alone or with either of the two other

Markers selected for on mating plates	No. of colonies tested	Marker present		
		leu$^+$	*his*$^+$	*gal*$^+$
leu$^+$	100	100	92	100
leu$^+$ *his*$^+$	60	60	60	52
his$^+$ *gal*$^+$	100	2	100	100
leu$^+$ *gal*$^+$	30	30	30	30
leu$^+$ *his*$^+$ *gal*$^+$	10	10	10	10

Table 11. Coinheritance of F-prime markers in progeny from a cross of ED1294 with ED2620.
Single well separated standard-size colonies picked from selective minimal plates were patched on minimal agar of the same composition and incubated for 24 hours. They were then replicated on selective minimal agar on which the presence of the different episomal markers could be detected. Replicas were incubated for 3 to 4 days. All plates contained streptomycin.

markers. However the presence of leu$^+$ in an autonomous state in the "triple" strain was confirmed among clones in which either gal$^+$ or his$^+$ were lost by spontaneous segregation; when such Gal$^+$Leu$^+$ or His$^+$Leu$^+$ clones were treated with acridine orange, simultaneous curing of both markers in each pair occurred although very rarely. Moreover, when such "doubles" were crossed with ED1308, leu$^+$ was transferred with a frequency 2 to 5% of that which with either gal$^+$ or his$^+$ were transferred. It was always inherited alone in the progeny. The apparent absence of Leu$^+$ transfer from the "triple" strain therefore may be a consequence both of the poor donorability of KLF4 itself, so its transfer is prevented by the very efficient transfer of gal$^+$ and his$^+$, and also of the possibility that if transferred with either his$^+$ or gal$^+$ it could then be segregated out in the new recipient.

The results described suggest that in this particular system, the gal$^+$, his$^+$ and leu$^+$ genes carried originally on three different F-prime factors can coexist stably in the same recipient. The evidence from the independent segregation and transfer suggests that they are at least part of the time autonomous.

F. Discussion

The results described in the first part of this section show that under the conditions used, mutagenesis of the F-prime strains did not allow the isolation of mutants F-prime factors able to cohabit. In fact the results were qualitatively very similar to those obtained in section I for the same pair of F-prime factors in the absence of mutagenesis. It is possible that this lack of success is due to the impossibility of isolating such mutants. It is possible that a mutation in an F-prime factor affecting the incompatibility function would also affect replication of the F-prime factor in the autonomous state and would not

allow this sort of mutant to be detected. If so, mutants of this sort may be only detected in the integrated state (Hfr) where F replicates passively with the chromosome.

In contrast the results presented in the second half of this section show what seems to be the isolation of mutants of ED1314 after mutagenesis with NTG, in which two different autonomous F-prime factors seem to coexist stably. This conclusion is based on the following evidence. (a) Spontaneous and independent segregation and curing of the two markers carried by the F-prime factors is observed. (b) In conjugation experiments using the mutant harbouring these two F-prime factors as donor in matings with a recA recipient, independent transfer of both F-prime factors occurs.

The fact that independent, and more rarely, simultaneous loss of the two episomal markers in the "double" strain occurs, and the absence of progeny inheriting both gal$^+$ and his$^+$ together in crosses of the "double" Gal$^+$His$^+$ strain with a recA recipient, tends to exclude the possibility that both episomal markers are physically joined; in such a model it would be expected that this hybrid episomal structure would also be found in the new recipient. Also if either or both of these episomal markers can integrate into the chromosome, it must be a rare event, since the host strain is recA. Thus at least in the majority of cells both gal$^+$ and his$^+$ co-exist in a state in which they are autonomous from the chromosome. The possibility of dealing with a mixed population carrying either episomal marker in the autonomous state was ruled out by segregation and transfer experiments.

The reasons for believing that the observed stability of the coexisting F-prime factors is due to a NTG-induced mutation in the host rather than one or other of the F-prime factors involved, is that cells cured of both F-prime factors can be reinfected and the "double" phenotype can be reconstituted at the

expected high frequency. Moreover, mutant strains already harbouring F*gal*$^+$ and F*his*$^+$ seems to be able to accept a third F-prime factor (KLF4) which also seems to replicate autonomously in the presence of the two resident F-prime factors. Also colonies of this mutant show a mucoid morphology absent in the non-mutagenized strain from which it was derived. This mucoid phenotype is expressed in this mutant whether the F-prime are presents or not, is not transferred by either of the F-prime factors involved in this study and does not seem to affect any function other than allowing stable cohabitation of two autonomous F-prime factors.

The results discussed suggest an active participation of the host cell in the expression of incompatibility between autonomous F-prime factors. However the information obtained from these experiments does not help to distinguish between the two hypotheses which have been devised to explain incompatibility and which have been discussed in the Introduction.

SECTION III

ISOLATION AND CHARACTERIZATION OF DEFECTIVE F-PRIME FACTORS

A. Introduction

The object of these experiments was to isolate F-prime factors defective in the incompatibility function. Since in an Hfr strain, according to Campbell's model, F is continuous with the bacterial chromosome, in the formation of F-prime factors, a piece of F can sometimes be left in the host chromosome during its transition from the integrated (Hfr) state to the autonomous state (Adelberg and Burns, 1960). Such F-prime factors might still be detected if the missing piece does not contain any gene(s) essential for the autonomous replication of the sex factor. A series of F-prime factors were therefore isolated and examined for the loss of the incompatibility function.

In section I of this thesis it was mentioned that both KLF1 and KLF4, in contrast to the rest of the F-prime factors used, do not restrict the growth of the female specific phage ØII. A possible explanation may be that the genes responsible for the full expression of these properties were lost during the formation of these F-prime factors. Therefore it was considered worthwhile to isolate further F-prime factors from the same Hfr strain (HfrH AB259) from which KLF1 and KLF4 were isolated to test whether such F-prime factors were also defective with respect to these properties, and also whether some of them would be defective in incompatibility. It was found that some are indeed defective but their formation can not be explained only by the simple model described above.

The method used in these experiments was that of Low (1968), in which after a mating between an Hfr and an F^- *recA* strain, F-prime factors carrying early, late, and both early and late chromosomal markers from the donor Hfr were selected for. The expectation was that among the F-prime factors carrying either early

or late markers would be some that would carry deletions of one end or the other of the integrated F as found by Low (1968). The formation of these defective F-prime factors has been schematized by Scaife (1967).

HfrH AB259 is a prototrophic streptomycin sensitive strain in which the origin of transfer is located between *thr*$^+$ and *ura*$^+$ on the genetic map, and which transfers chromosomal genes in the order: O-*thr*$^+$-*leu*$^+$.....*met*$^+$-*ura*$^+$-F. The strain used as recipient in these experiments was KL132, an F$^-$ *recA ura thr str* strain. Therefore the following three different classes of F-prime factors could be selected: (a) a class carrying early markers (F-*thr*$^+$...), (b) a class carrying late markers (F-*ura*$^+$...) and (c) a class carrying both early and late markers (...*ura*$^+$-F-*thr*$^+$...). On a simple model one could expect to find defective F-prime factors carrying deletions in either end of F, among those belonging to classes (a) and (b), assuming that replication genes are not deleted. In contrast F-prime factors of class (c) are a type of control since this class of F-prime factors would be expected to carry a complete F. One would also expect that the correlation of the properties of these defective F-prime factors might allow the mapping of the missing functions on the integrated F.

B. Results

Equal volumes of exponentially growing cultures in L-broth of AB259 and KL132, each at about 2 x 10^8 cells per millilitre, were mixed and incubated at 37°C. The mating was interrupted after 1 hour in order to prevent the entry of the *recA*$^+$ gene from the donor and 0.1 ml. samples of 10^0 to 10^2 dilutions of the mating mixture were spread onto separate selective minimal agar plates containing streptomycin, for the selection of *ura*$^+$, *thr*$^+$ and *ura*$^+$ *thr*$^+$.

After 5 days incubation colonies of different sizes showed up on the three types of minimal plates used. Colonies of each size from each class were purified by two successive single colony isolations on the original selective media, spread with a drop of L-broth. This was especially necessary in the case of the Ura^+ progeny, which grew very poorly. After purification single colonies were grown in selective liquid minimal media in order to check the inheritance of unselected markers from the donor Hfr strain. These purified clones were highly UV-sensitive, showing that they had retained the $RecA^-$ phenotype.

Table 12, shows the frequency of the progeny on the different selective plates used. It also shows the distribution of the unselected markers among these progeny. In the progeny obtained when selecting for Ura^+, 15 out of 30 clones tested had also inherited Thr^+; the rest had inherited Ura^+ alone. In contrast among the Thr^+ progeny only 6 out of 50 clones tested had inherited Thr^+ alone; the others were either Leu^+ or Ura^+ or both Leu^+ and Ura^+. The majority of the Thr^+ clones tested, 33 out of 50, had also only inherited Leu^+. Only 11 out of 50 had inherited both Ura^+ and Thr^+ and among these 8 were also Leu^+. Among the progeny obtained when selecting for both Ura^+ and Thr^+ simultaneously only 5 clones were tested for coinheritance and among these, 4 showed this phenotype while the other 1 had coinherited Leu^+. The pattern of coinheritance therefore seems to be dependent on the markers selected for.

Since all the clones were highly UV-sensitive and therefore presumably $RecA^-$ it was assumed that the transferred markers had not become integrated into the host chromosome but were present extrachromosomally. In order to replicate, these extrachromosomal segments must therefore presumably carry at least that segment of F that allows F to replicate itself. Evidence for the presence of F was obtained by testing these presumptive F-prime strains with phages MS2 and ØII. In addition, in order to confirm the replicative autonomy

Selected donor marker	Frequency of colonies arising on selective minimal agar (a) ($\times 10^{-4}$)	Number of purified clones tested	Number of colonies showing the phenotype				
			Ura^+	Ura^+Thr^+	Thr^+	Thr^+Leu^+	$Ura^+Thr^+Leu^+$
ura$^+$	3.3	30	15	15	0	0	0
thr$^+$	0.8	50	0	3	6	33	8
ura$^+$ *thr*$^+$	0.4	5	0	4	0	0	1

Table 12. Frequency of colonies arising on minimal agar selecting for *ura*$^+$, *thr*$^+$ and *ura*$^+$ *thr*$^+$ progeny, and distribution of unselected markers. Exponential cultures in L-broth with about 2×10^8 cells/ml of both AB259 and KL132 were mated 1:1 at 37°C. After 1 hour the mating was interrupted by violent agitation and 0.1 samples of 10^0, 10^1 and 10^2 dilutions were plated onto minimal agar containing streptomycin. Plates were incubated at 37°C for 5 days. Single well separated colonies of each type were purified by two successive single colony isolations on the same selective media. Single purified colonies were then grown in selective liquid minimal media to check coinheritance of unselected markers.

(a) Colonies per 100 cells of either parent input.

of F, these purified clones were also tested for their ability to give rise to segregants both spontaneously and upon acridine orange treatment. Their transfer ability was tested in crosses with ED1490, a spontaneous Spc^r derivative of KL132. These data are summarized in Table 13, where it can be noted that the transfer abilities among the presumptive F-prime factors tested differ strikingly.

It can be seen that the presumptive F-prime factors tested can be grouped in three categories: typical F-primes, non-donor males, and females.

(i) Typical F-primes: these were characterized by their ability to mediate their own transfer with high frequency in crosses with an F^- recA recipient and also by their sensitivity to phage MS2 and resistance to phage ØII. The extrachromosomal state of these F-prime factors was further indicated by the segregation of episomal markers either spontaneously or after treatment with acridine orange. Both presumptive $Fthr^+$ clones tested, all three $Fura^+thr^+leu^+$, 4 out of 11 $Fura^+thr^+$ and 9 out of 12 $Fthr^+leu^+$ fell in this category.

(ii) Non-donor males: all eleven presumptive $Fura^+$ tested, 7 out of 11 $Fura^+thr^+$ and 1 out of 12 $Fthr^+leu^+$ fell in this category. All of them failed to mediate their own transfer in crosses with a recA recipient, but appeared to make normal F-pili, since they were sensitive to phage MS2. The presence of F in these strains was further indicated by the fact that all members of this group inhibit the growth of phage ØII.

In order to test whether or not members of this class were Hfr strains, they were crossed with ED2742 (F^- leu his gal lac thr ura spc) for 3 hours, in which $Ura^+(Spc^r)$, Thr^+ (Spc^r) and Leu^+ (Spc^r) were selected. No progeny which had inherited either of these markers could be found. Since the recipient strain is $recA^+$, this observation seems to suggest that either these strains are not Hfr strains, or if they are, they may be defective in transfer. Besides the

Phenotype of presumptive F'-factor	Number of colonies in each class	Transfer ability (a)	Response to phage (b) MS2	ØII	Segregation (c)
Ura^+	11	-	S	R	-
Thr^+	2	+	S	R	+
$Thr^+ Leu^+$	1	-	S	R	-
$Thr^+ Leu^+$	2	-	R	S	-
$Thr^+ Leu^+$	9	+	S	R	+
$Ura^+ Thr^+$	4	+	S	R	+
$Ura^+ Thr^+$	7	-	S	R	-
$Ura^+ Thr^+ Leu^+$	3	+	S	R	+

Table 13. Properties of presumptive F-prime factors.

(a) Exponential cultures in L-broth of each of the presumptive F'-strains tested and of ED1490, each at about 2×10^8 cells/ml., were crossed 1:5 at 37°C. After 30 minutes the matings were interrupted by violent agitation and 0.1 ml. samples of 10^0 to 10^4 dilutions were plated onto spectinomycin supplemented selective minimal agar for the selection of progeny inheriting episomal markers from the donor strains and incubated at 37°C for 5 days. Controls for reversion were carried out simultaneously with each mating. + = Normal frequency of transfer (75-120% of the donor input). - = No transfer ($< 10\text{-}5$).

(b) overnight cultures in L-broth from single colonies of each of the presumptive F'-strains were tested for sensitivity to phages MS2 and ØII (see Materials and Methods). S = Sensitivity, R = Resistance. Both parental strains AB259 and KL132 were used as controls; they are $MS2^s ØII^r$ and $MS2^r ØII^s$ respectively.

(c) samples of each of the presumptive F'-strainswere tested for spontaneous segregation and also were treated with different concentrations of acridine orange as described in Materials and Methods. + = segregation ($< 1\%$) of any of the episomal markers. - = No segregation.

observed lack of fertility in crosses with either recA or recA$^+$ recipients, the members of this class differed from the typical F-primes mentioned above in that neither spontaneously nor after treatment with acridine orange they give segregants. An alternative possibility is that these strains are now F$^+$ or F-prime strains in which the episomal markers are now recombined into the chromosome. A third possibility is that these strains of the F-prime factors they carry are acridine orange-insensitive.

(iii) Females: 2 out of 9 of the presumptive Fthr$^+$leu$^+$ tested are assigned to this category on the basis of their lack of transfer ability, the failure to give segregants for either marker and their resistance to phage MS2 and sensitivity to phage ØII. The formation of these two strains may be explained by assuming (a) that in spite of the RecA$^-$ phenotype of these strains the inherited donor markers but not F have become integrated into the host chromosome by a mechanism other than the Rec system, or (b) that these strains have inherited a defective F in which the genes for the properties looked for in these tests are absent, or (c) that these strains represent rare cases of reversion for these two markers in the recipient strain.

Since none of the non-donor males (ii) have been conclusively proved to be defective F-prime factors, the study of their properties cannot be used to relate the supposedly missing functions with their possible location in the F segment. In further experiments both typical F-prime factors (i) and non-donor males (ii) will be identified by their serial numbers and by the markers inherited from the parent HfrH strain (see Table 14). Representatives of both categories were selected to study their incompatibility properties.

Markers inherited from parental HfrH (AB259)	Strain number	Transfer ability	Segregation
(i) Typical F-primes			
thr$^+$	ED1559	+	+
"	ED1561	+	+
ura$^+$ *thr*$^+$ *leu*$^+$	ED1543	+	+
"	ED1547	+	+
"	ED1557	+	+
ura$^+$ *thr*$^+$	ED1516	+	+
"	ED1518	+	+
"	ED1533	+	+
"	ED1583	+	+
thr$^+$ *leu*$^+$	ED1530	+	+
"	ED1531	+	+
"	ED1539	+	+
"	ED1542	+	+
"	ED1549	+	+
"	ED1550	+	+
"	ED1554	+	+
"	ED1560	+	+
"	ED1564	+	+
(ii) Non-donor males			
ura$^+$	ED1517	-	-
"	ED1521	-	-
"	ED1523	-	-
"	ED1525	-	-
"	ED1526	-	-
"	ED1527	-	-
"	ED1578	-	-
"	ED1580	-	-
"	ED1582	-	-
"	ED1584	-	-
"	ED1585	-	-
ura$^+$ *thr*$^+$	ED1519	-	-
"	ED1520	-	-
"	ED1522	-	-
"	ED1524	-	-
"	ED1581	-	-
"	ED1588	-	-
"	ED1590	-	-
thr$^+$ *leu*$^+$	ED1491	-	-

Table 14. Strain numbers given to presumptive F-prime factors isolated from AB259(HfrH) x KL132(F$^-$ *recA*) crosses. For details see text and Table 13 .

+ = Normal frequency of transfer; spontaneous segregation.

- = No transfer ($< 10^{-5}$); no segregation.

C. The isolation and characterization of Gal$^+$Ura$^+$Thr$^+$ strains from F8(gal$^+$) and Fura$^+$thr$^+$

F$^-$ phenocopies of representatives of groups (i) and (ii) were used as recipients in 1:5 matings with exponential cultures of ED1232 (Fgal$^+$). Matings were allowed to proceed for 30 minutes before interruption. Dilutions were plated onto selective minimal agar containing streptomycin and spread with a drop of L-broth. Plates were incubated for up to 5 days. The results of these matings are shown in Table 15.

From these results it can be observed that the presumptive F-prime factors tested can be grouped in two categories on the basis of their recipient abilities: (a) those which show inheritance of gal$^+$ at the efficiency expected from an F-prime strain in F$^-$ phenocopy state; ED1517(ura$^+$), ED1520(ura$^+$), and ED1516(ura$^+$ thr$^+$), and (b) those which show no inheritance of gal$^+$; ED1491(thr$^+$ leu$^+$) and ED1530(thr$^+$ leu$^+$).

To explain the results observed with members of class (b) one may assume that either they can not be converted in F$^-$ phenocopies when grown to late stationary phase and that in both ED1491 and ED1530, even in this condition, surface exclusion to the superinfecting Fgal$^+$ is fully expressed, or more probably, incompatibility between these F-prime factors is responsible for the absence of progeny inheriting markers from both episomes. This behaviour does not seem to be related to the transfer ability of the recipient, it will be noted that none of the ura$^+$ presumptive F-prime factors fall into this class.

Whether or not ED1517 (ura$^+$), ED1520(ura$^+$ thr$^+$) and ED1516(ura$^+$ thr$^+$) lack surface exclusion was tested by using these presumptive F-prime strains in exponential phase as recipients. Such matings were carried out and the expected high reduction in the frequency of gal$^+$ inheritance was observed, which indicates that surface exclusion had not been lost in these presumptive F-prime factors.

Recipient	Markers selected (a) ura$^+$ gal$^+$	ura$^+$ thr$^+$ gal$^+$	thr$^+$ leu$^+$ gal$^+$
(i) Typical F-primes			
ED1516 (ura$^+$ thr$^+$)	9	15	$< 10^{-4}$
ED1530 (thr$^+$ leu$^+$)	$< 10^{-4}$	$< 10^{-4}$	$< 10^{-3}$
(ii) non-donor males			
ED1517 (ura$^+$)	12	$< 10^{-4}$	$< 10^{-4}$
ED1520 (ura$^+$ thr$^+$)	19	17	$< 10^{-4}$
ED1491 (thr$^+$ leu$^+$)	$< 10^{-4}$	$< 10^{-4}$	$< 10^{-3}$

Table 15. Infection of presumptive F-prime strains with F8(gal$^+$).
Late stationary cultures in L-broth of each of the presumptive F-prime strains used in this experiment were diluted to 2 x 10^8 cells./ml. and crossed 1:5 with exponential cultures of ED1232 as donor, at the same concentration. The matings were interrupted after 30 minutes and 0.1 ml samples of 10^0 to 10^4 dilutions of the mating mixtures were plated onto selective minimal agar containing streptomycin and incubated for up to 5 days.

(a) Average of colonies per 100 donor cells in the mating.

After the Rec$^-$ phenotype of single purified colonies from each of the progeny showing inheritance of Gal$^+$ (Table 16) had been confirmed, such clones were tested for segregation and curing by acridine orange. All segregated Gal$^-$ clones either spontaneously or after acridine orange treatment, but only those colonies selected from Fgal$^+$ x ED1516(Ura$^+$Thr$^+$) crosses segregated both sets of episomal markers. In no cases did acridine orange increase this segregation frequency. Loss of both sets of episomal markers together was never observed.

These "double" clones were next used as donors in 1:5 matings with ED1490 (F$^-$ recA ura thr leu spc) for 30 minutes. After interruption dilutions of the mating mixtures were plated onto spectinomycin-supplemented selective minimal agar for the selection of ura$^+$ and gal$^+$ progeny.

As can be seen from the results shown in Table 16 only the presumptive "double" Gal$^+$Ura$^+$Thr$^+$(F8/ED1516) transfers both sets of episomal markers with high frequency, although this frequency is not as high as with the single F-primes used as controls. Tests for coinheritance revealed only two classes of progeny; all those which had inherited gal$^+$ were ura$^-$thr$^-$ and all those inheriting ura$^+$thr$^+$ were gal$^-$; none of several hundred colonies tested, had inherited gal$^+$ and ura$^+$thr$^+$ together, although on the basis of the yield of Gal$^+$ and Ura$^+$Thr$^+$ progeny, this class might have been expected. However since both gal$^+$ and ura$^+$thr$^+$ were not selected for simultaneously in these matings, the possibility that they could have been transferred together is not completely ruled out. In the other two strains tested Gal$^+$Ura$^+$Thr$^+$(F8/ED1520) and Gal$^+$Ura$^+$ (F8/ED1517) only gal$^+$ was transferred. The same results were observed when these crosses were repeated. This seems to indicate either that if ura$^+$thr$^+$ (ED1520) and ura$^+$(ED1517) are indeed present episomally in their respective strains, their transfer deficiency cannot be complemented by F8, the alternative is that ura$^+$ and ura$^+$thr$^+$ in these "non-donor males" are chromosomal.

Donor phenotype	Selected marker[a] ura$^+$	gal$^+$
Gal$^+$ (F8)	$< 10^{-4}$	53
Ura$^+$Thr$^+$ (ED1516)	50	$< 10^{-4}$
Gal$^+$Ura$^+$ (F8/ED1517)	$< 10^{-4}$	35
Gal$^+$Ura$^+$Thr$^+$ (F8/ED1520)	$< 10^{-4}$	43
Gal$^+$Ura$^+$Thr$^+$ (F8/ED1516)	47	40

Table 16. Donorability of presumptive "double" strains.
Exponential cultures in L-broth of each of the strains carrying the "double" phenotype and of the parental F-prime strains were used as donors in 1:5 matings with ED1490 (F$^-$ recA) for 30 minutes. All cultures were at about 2 x 10^8 cells per ml. After interruption, 0.1 ml. samples of 10^0 to 10^4 dilutions were plated onto selective minimal agar containing spectinomycin. Plates were incubated for up to 5 days.

(a) Colonies per 100 donor cells in the mating.

The absence of a class of progeny inheriting markers from both episomes simultaneously in the crosses using the gal$^+$ ura$^+$ thr$^+$ (F8/ED1516) strain as donor might be explained by assuming that the two sets of markers must be transferred independently to different recipient cells. The possibility that they could be transferred together but either of them be segregated out because of incompatibility in the new host is less probable, since, if this cohabitation is due, as it seems to be, to a mutation in the Fura$^+$ thr$^+$ (ED1516), both F-prime factors should coexist stably in the new recipient. However in order to test this possibility a cross between a "double" Gal$^+$Ura$^+$Thr$^+$ and an F$^-$ recA$^+$ recipient was carried out. The rationale was that if two episomes are transferred together to the same recipient, recombination with the host chromosome could rescue episomal markers which in a recA recipient would be segregated out. The recipient used in this cross was ED2742 (F$^-$ ura thr gal spc) strain. The same two classes of progeny were found as before, but again no class inheriting markers from both episomes together was detected, even on plates on which ura$^+$ and gal$^+$ were selected for simultaneously.

Since 4 independently isolated Fura$^+$ thr$^+$ (ED1516, ED1518, ED1533 and ED1583) tested under the same conditions turned out to coexist with F8 it seems that this impaired incompatibility is a common property of F-prime factors of this type rather than a consequence of the selection of a variant among them. However the possibility of a mutation occurring in these F-prime factors during their formation is not ruled out, although its occurrence as a deletion in F seems improbable since the presence of both ura$^+$ and thr$^+$ in these F-prime factors suggests the existence of a complete F.

D. The isolation and characterization of His$^+$Ura$^+$Thr$^+$ strains from F30(his$^+$) and Fura$^+$thr$^+$

The next step was to find out whether or not Fura$^+$thr$^+$ (ED1516) could also coexist with other F-prime factors. F30(his$^+$) and KLF4(thr$^+$leu$^+$pro$^+$) were tested for cohabitation with Fura$^+$thr$^+$ following the same procedure as with F8. Of these two F-prime factors, when the same criteria are applied as with F8, only F30 seems to coexist stably with Fura$^+$thr$^+$. In F30 x Fura$^+$thr$^+$ crosses, progeny showing inheritance of his$^+$ were found at a frequency of about 10-20% of the donor input. When purified single colonies from this mating were tested for segregation, each episome was lost at a low frequency (1%). Treatment with acridine orange neither increase the frequency of loss of either marker nor showed any curing of both episomes together. When populations originating from His$^+$Ura$^+$Thr$^+$ clones were used as donors in crosses with both ED1490 (F$^-$recA) and ED2742 (F$^-$recA$^+$) only two classes of progeny were found; those which had inherited his$^+$ were Ura$^-$Thr$^-$ while those inheriting ura$^+$thr$^+$ were His$^-$. No class showing the His$^+$Ura$^+$Thr$^+$ phenotype was found although simultaneous selection for both sets of markers was included. These results are therefore analogous to those described in the previous section. From these results it is possible to conclude that both Fhis$^+$ and Fura$^+$thr$^+$tra$^+$ can replicate in and transfer independently from the same cell.

In contrast, when fertile cultures of a strain harbouring KLF4 (Fthr$^+$leu$^+$pro$^+$) was used as donor it was not even possible to isolate any Fura$^+$thr$^+$tra$^+$ progeny that had inherited either leu$^+$ or pro$^+$. It could be that either KLF4 had not been able to infect the recipient, or that if it had, incompatibility then followed immediately after infection. The first alternative seems less probable since in spite of the poor donorability of KLF4 one would have expected some inheritance of either leu$^+$ or pro$^+$.

E. Discussion

By using an F⁻ recA recipient in crosses with an HfrH strain, it was possible to isolate presumptive F-prime factors which carry early, late, and both, early and late Hfr markers. These presumptive F-prime factors may have been formed in the Hfr population by a rare "loop-out" of the sex factor together with either distal, or proximal, or both ends of the chromosome according to the model elaborated by Broda et al (1964) (see also Scaife, 1967). All the presumptive F-prime factors whose isolation is described in this section fall in two categories. Presumptive F-prime factors belonging to the first category segregate spontaneously although at a low frequency and are able to mediate their own transfer very efficiently in crosses with an F⁻ recA recipient. They are also sensitive to phage MS2 and resistant to phage ØII. These therefore, seem to represent typical F-prime factors. On the basis of the episomal markers they carry, these F-prime factors can be grouped in two classes; one class carries early markers (thr⁺; thr⁺ leu⁺) and a second class which carries both early and late markers together (ura⁺ thr⁺; ura⁺ thr⁺ leu⁺). Typical F-prime factors carrying late (ura⁺) marker(s) alone could not be isolated.

Presumptive F-prime factors belonging to the second category have inherited early, late, and both, early and late markers from the parental HfrH strain. However the inherited markers are neither lost spontaneously nor after acridine orange treatment which suggests that the markers involved are not carried autonomously, but have become integrated into the chromosome in spite of the RecA⁻ phenotype of these strains. Members of this category are sterile when crossed with either a recA or a recA⁺ recipient, although they are sensitive to phage MS2 and resistant to phage ØII. This seems to suggest that members of this category may be defective in transfer. Assuming that these non-donor

males are defective F-prime factors, their formation cannot be satisfactorily explained by a simple model for the origin of F-prime factors. The observation that members of this category carry markers on both sides of the sex factor, seems to exclude the possibility that this lack of donorability might be due to a deletion in either end of the sex factor which occurred during their formation, since one would have expected these presumptive F-prime factors to carry a complete F. However it must be pointed out that while only 1 out of 12 presumptive F-prime factors carrying only early markers (thr$^+$;thr$^+$leu$^+$) may be transfer defective, all 11 presumptive F-prime factors carrying only a late marker (ura$^+$) seem defective too. This distribution may well suggest that genes responsible for transfer may be located in these presumptive F-prime factors closer to the early markers. Low (1968) has described the isolation from the same HfrH strain of donor and non-donor classes of F-prime factors, although his non-donor class of F-primes were resistant to both MS2 and ØII phages. Examples of this particular class of F-prime factors were not found in this study. Another point of difference is that the transfer-proficient F-prime factors described here are better donors than the ones that he described (KLF1 and KLF4).

Among members of the first category there is one class (Fura$^+$thr$^+$) which can coexist stably with either F8(gal$^+$) or F30(his$^+$) but not with KLF4 (Fthr$^+$leu$^+$pro$^+$). The conclusion that both F-prime factors of each pair can replicate autonomously, at least part of the time, is based on the following evidence: (a) independent segregation of episomal markers from either F-prime factor occurs. The low frequency of this segregation rules out the possibility that the culture consists of a mixed population of single F-prime strains, and (b) conjugation experiments using strains presumably harbouring either pair of F-prime factors as the donor in crosses with a recA recipient show the independent transfer of both F-prime factors with high efficiency.

The discovery that three other independently isolated F-prime factors carrying the same markers (F*ura*$^+$ *thr*$^+$) can coexist with F8(*gal*$^+$), as determined by genetic criteria, strongly suggests that this is a common property of this class of F-prime factors rather than a singular property of a particular member of it. The fact that other classes of F-prime factors isolated under the same conditions and simultaneously with those F*ura*$^+$ *thr*$^+$ do not give "doubles" with F*gal*$^+$ suggests that in these F-prime factors an alteration in the genetic control of incompatibility has occurred. However since *ura*$^+$ and *thr*$^+$ are markers on opposite sides of the integrated sex factor in the HfrH, their presence in these F-prime factors makes it unlikely that such a change could be due to a deletion in F. However the fact that KLF4, unlike F*gal*$^+$ and F*his*$^+$, is unable to give "doubles" with the F*ura*$^+$ *thr*$^+$ strain described here, seems to exclude the possibility that a mutation affecting the expression of incompatibility has occurred in the F*ura*$^+$ *thr*$^+$, since one would expect that this mutation would also allow this F-prime factor to coexist stably with KLF4. One possible explanation is that this incompatibility is due in some way to their common ancestry from HfrH.

In section I it was shown that both KLF1 and KLF4 are sensitive to the female specific phage ØII. Although the same parent strains and the same method for the isolation of these class of defective F-prime factors (Low, 1968) were used in this section, this class of defective F-prime factors was not isolated. Also the "doubles" given by KLF1 and KLF4 (section I) with either F*gal*$^+$ or F*his*$^+$ are different from those obtained in this section; thus while neither KLF1 nor KLF4 showed segregation and were always transferred together with either *gal*$^+$ or *his*$^+$, the "doubles" described in this section show independent segregation and independent transfer of episomal markers. On the other

hand although both KLF4 or F*ura*$^+$ *thr*$^+$ seem to be compatible with either F*gal*$^+$ or F*his*$^+$, they are not compatible with each other.

Although some of the F-prime factors which have been isolated in this section seem to coexist stably with either F*gal*$^+$ or F*his*$^+$, they have not been proved to be mutants defective in the incompatibility function. The observation that these F-prime factors seem to carry a complete sex factor, excludes the possibility that this apparent lack of incompatibility may be due to a deletion in the sex factor.

CONCLUSIONS.

The primary object of this research was to get information which would help to the understanding of incompatibility between autonomous F-prime factors in Escherichia coli. Three different approaches were followed. Firstly, several different F-prime factors were studied for their ability to cohabit in a multiple auxotrophic recA strain. Secondly, mutagenesis of a pair of F-prime factors and of the recipient strain was attempted in order to isolate mutants in which incompatibility could be impaired. An thirdly, a set of F-prime factors were isolated in the hope that some of them might be defective in the incompatibility function.

In the first section of this thesis, stable "double" strains carrying episomal markers from two different F-prime factors were isolated. However the genetical study of their properties did suggest that in no case did these "double" strains represent clear cases of stable cohabitation between two autonomous F-prime factors. None of the F-prime factors involved in the formation of these "doubles" seemed to have lost the property of inhibiting the autonomous establishment of a second autonomous F-prime factor in the same cell.

Mutant F-prime factors defective in the incompatibility function could not be obtained after mutagenesis of two different F-prime strains (section II), nor were they found amongst a variety of newly isolated F-prime factors from an HfrH strain (section III). However a particular class of the latter seemed to show impaired incompatibility. In contrast mutagenesis of the recipient strain (section II), allowed the isolation of a mutant in which two and even three different F-prime factors seemed, by genetic criteria, to coexist stably. The principal conclusion obtained from these studies was the confirmation of the participation of the host strain in the expression of incompatibility between

autonomous F-prime factors. Palchoudhury and Iyer (1971) described stable cohabitation of two autonomous F-prime factors in a DNA-t^s43 mutant strain unable to replicate its chromosome at high temperature. These authors suggested that this mutation involves a component of the cell membrane which allows this mutant to permit two autonomous F-prime factors to replicate stably. The experimental results described in this section suggested that a mutational event might have increased the capacity of the cell to allow two different autonomous F-prime factors to replicate stably. The isolation of this mutant tends to support the model of "attachment-sites" postulated by Jacob et al (1963). However it does not exclude the applications of a different mechanism for the control of incompatibility (Pritchard et al., 1969).

Although the results presented in this thesis do not help to decide between the models which have been proposed to explain incompatibility between isogenic or closely related sex factors, the application of the methods employed in these sections in a more exhaustive fashion and the use of different Hfr strains, may lead to the isolation of more strains in which cohabitation apparently occurs, which could provide a better understanding of the mechanism of incompatibility. Furthermore, the demonstration of independent replication of episomes by the method described here, and by other methods would provide an essential tool for the genetic analysis by complementation of sex factors mutants in the replication function.

ACKNOWLEDGEMENTS

I should like to thank Dr. Paul Broda for his patient supervision and encouragement during the course of this work and for his criticism in the writing of this thesis. I also thank D.J. Finnegan and Dr. N.S. Willetts for their valuable comments on the manuscript. I am also grateful to Professor M.R. Pollock for the opportunity of doing this work in the Dept. Molecular Biology, University of Edinburgh.

I wish to acknowledge the receipt of a scholarship from the Instituto Venezolano de Investigaciones Científicas.

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