GENETIC ANALYSIS OF A DOUBLE MALE STRAIN OF *ESCHERICHIA COLI* K12[1]

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ABSTRACT

The behavior of a double male strain of *Escherichia coli* K12 has been compared to that of its parents and a primary F′ strain carrying F14 in order to determine whether the genome of the double male, responsible for the double origin mode of gene transmission, is composed of one or two linkage groups. F-prime plasmids carrying *leu* and *pyrB* and *argG* and *metC* have been detected following mating an F⁻ *recA*⁻ recipient with the double male. Their existence strongly supports the contention that the double male is composed of a single linkage group with two integrated sex factors. Data from acridine orange curing experiments places the frequency of double male cells in the population of growing cells in the two-chromosome configuration as less than one percent. Evidence for a unique origin and terminus of DNA replication deduced by the density labelling and transduction of double male DNA supports the contention that the double male is best considered a cell with a single chromosome carrying two integrated F plasmids.

FROM a cross between two Hfr strains of *Escherichia coli* K12 (one an F⁻ phenocopy) transconjugants* were selected which had inherited markers closely linked to each of the sex factors of the parents (CLARK 1963). One of these, JC182, had inherited the ability to transfer DNA from two points of origin and to sire recombinants inheriting the sex factor characteristic of either parent. On this basis the strain in question was labelled a double male. Since recessive markers from both parents were expressed and the average DNA content of each nuclear body was found to equal that of the two parents, the double male, JC182, was considered to be haploid. Beyond that, however, the genetic nature of the double male was open to conjecture. In particular, is the genome made up of one or two linkage groups? If a single chromosome comprises the genome of the double male then the two F-plasmids must be thought of as lying at two places on the chromosome, in much the same manner as an *Escherichia coli* cell doubly lysogenic for phage P2 in which the prophages can lie at widely separated sites close to *his* and *metB* (CALENDAR 1970). If there are two chromosomes, each could carry a sex factor. These alternatives are displayed in Figure 1. Actually these

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* Transconjugant—a bacterial cell that has received genetic material from another bacterium by conjugation (NOVICK *et al.* 1973).

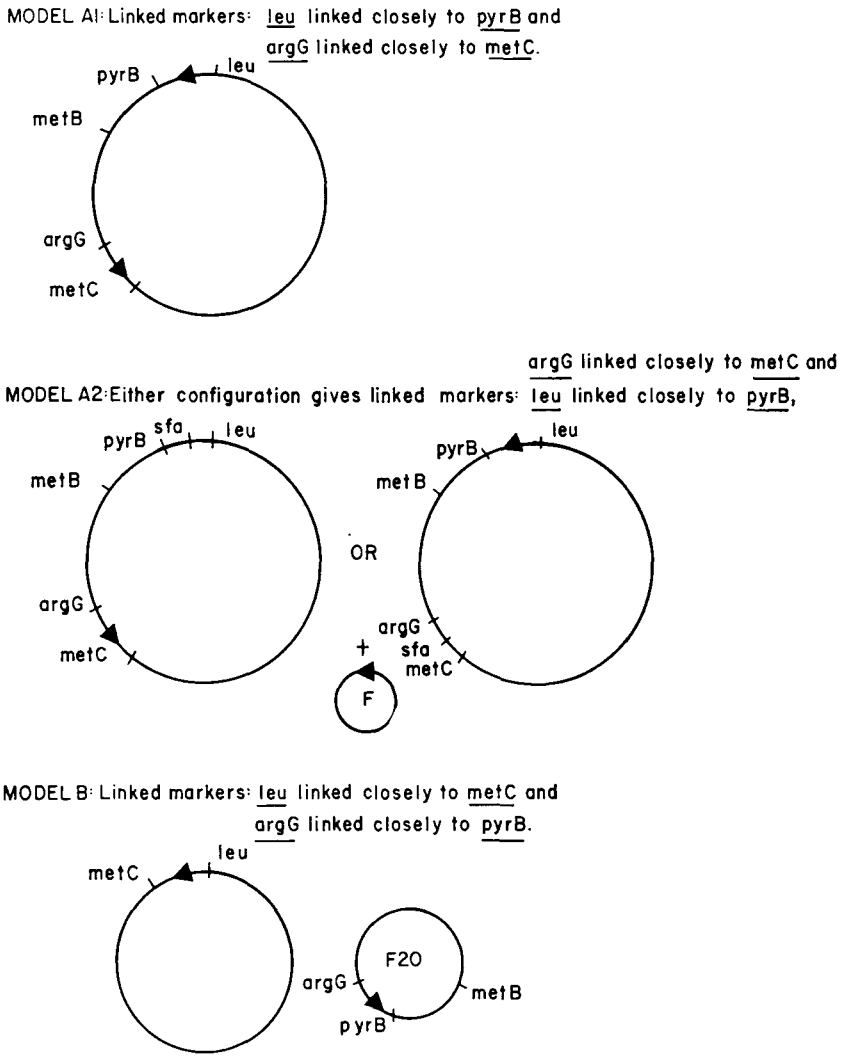

FIGURE 1.—Possible configurations for the genome of the double male strain of *Escherichia coli* K12. Model A1: One single linkage group with two integrated F plasmids. Model A2: One single linkage group with an autonomous F plasmid and chromosomal *sfa* locus and one integrated F plasmid. Two configurations are possible depending upon which F plasmid is autonomous. Model B: two linkage groups, one of which corresponds to the F20, F′ plasmid.

possibilities aren't mutually exclusive since a single reciprocal recombination event between two such extensive regions of homology as the F-plasmid could produce two chromosomes from one and *vice versa*. This report describes the techniques used to establish the presence of cells in the one- and two-chromosome configuration. Certain techniques were not employed because of their inherent limitations. Since P1 bacteriophage cannot transduce both the F-plasmid and markers linked to both sides of it (PITTARD 1965), such a transductional analysis

was not initiated. Marker frequency analysis of transconjugants in which the double male was the recipient was not successful. Probably this reflects the characteristic of conjugation that linkage relationships demonstrated by transconjugants are dependent upon those linkages in the donor and not in the recipient.

MATERIALS AND METHODS

Strains: Bacterial strains used in this study are set forth in Table 1. Symbols are explained in the footnote. The phage strain, P1 *vira*, used in the DNA replication experiments is the same as used in the work of WOLF, NEWMAN and GLASER (1968).

Media: Media are the same as described by CLARK (1963) with the exception of media used in the DNA replication experiments, which conform to those reported by WOLF, NEWMAN and GLASER (1968). All thymine-requiring strains were maintained on media containing 50 μg thymine/ml. Spectinomycin was the gift of DR. G. B. WHITFIELD of the Upjohn Company.

Bacterial crosses: Cultures for use in bacterial crosses were prepared by inoculating 10^8 cells from an overnight tube-grown culture into 10 ml L-broth contained in 125 ml side arm flasks. Cultures were aerated in reciprocating or rotary water baths at 37° until they reached the desired cell density as measured in a Klett-Sumerson colorimeter. Cells of the double male and its derivaties were grown to early stationary phase when used as donors (Ca 8×10^8 cells/ml). Donor and log-phase recipient cultures were mixed together to yield 2×10^7 donor cells and 2×10^8 recipient cells per milliliter in a final volume of 10 ml. Separation of mating pairs following mating was accomplished by vortexing 1.0 ml of the mating mixture for 60 seconds.

The mating types of patched colonies were determined by replicating 6-hour-old master patches onto minimal selective media seeded with lawns of appropriately marked F$^-$ Rec$^+$ or Rec$^-$ tester strains. In such tests, Hfr patches give rise to heavy zones of recombinant growth with F$^-$ Rec$^+$ bacterial lawns but no such growth with F$^-$ Rec$^-$ lawns; F$^+$ donor patches give rise only to scattered colonies on F$^-$ Rec$^+$ lawns; F′ donor patches give rise to heavy zones of growth with both F$^-$ Rec$^+$ and Rec$^-$ lawns when selections are made for markers carried on the F′ plasmid; for markers not on the F′ plasmid, Rec$^+$ bacteria give few colonies and Rec$^-$ lawns none.

Acridine orange treatment: An inoculum of 5×10^3 to 5×10^4 cells taken from an overnight culture of each strain in L-broth was added to 10 ml L-broth adjusted to pH 7.8 containing various concentrations of acridine orange. Cultures were incubated for various lengths of time at 37° in red or clear foil-covered Erlenmeyer flasks (to exclude light). Suitably diluted samples were plated for survivors on L-agar plates taking care to exclude light during the pipetting and plating operations. Colonies were picked, patched, and incubated overnight and these masters used to test for donor ability and growth factor requirements.

DNA replication: The uptake of the ^{14}C-thymine into TCA (trichloroacetic acid) insoluble material was examined in JC3327. The strain was grown overnight in complete minimal medium containing 1 μg thymine/ml. An inoculum was diluted 100-fold into 20 ml minimal medium with 1 μg thymine/ml and grown to log phase. The cells were harvested by filtration on sterile 142 mm Millipore filters (0.65 μm), washed with minimal medium containing thymine, and suspended in 20 ml minimal medium without isoleucine, valine and thymine. Ten ml samples were placed in separate tubes and to one isoleucine and valine were added to a final concentration of 100 μg/ml each. ^{14}C-thymine was added to both tubes to a final concentration of 1.25 μg/ml (21 μCi-mM). Filtration, washing, and suspension took less than two minutes. Samples of 0.5 ml were removed at 10 min intervals from 0 to 180 minutes, from the tubes incubated in a shaking rack at 37°. Cells were collected on Whatman GF/C filters, washed twice with 5 ml ice-cold TCA and two 20 ml washes of ice-cold distilled water. Filters were dried under a heat lamp and counted in a scintillation counter.

Temporal replication sequence of genetic markers: Experiments measuring the replication of genetic markers in the double male were performed using the techniques of WOLF, NEWMAN and GLASER (1968).

TABLE 1

Tabulation of strains utilized in the study of the double male

STRAIN	GENOTYPE* ara	leu	touA	proC	lac	tsx	gal	pyrF	his	purF	purC	recA	thyA	serA	lysA	metC	argG	spc	str	aroB	xyl	ilv	argH	metB	thi	pyrB	MATING TYPE AND TRANSMISSION GRADIENT	SOURCE OR DERIVATION
JC12		-		-	-	-				-											-			-			Hfr, O, argG, xyl,...F	Clark (1963)
JC158				-										-											-		Hfr, O, leu, proC,...F	Clark (1963)
JC182				-						-															-		Double Hfr, O, leu, proC,... and O, argG, xyl	Clark (1963)
JC1569		-							-		-						-		-					-			F⁻	Low (1968)
JC3327										-		-										-			-		Double Hfr, see JC182	JC182
JC3419		-							-								-	-	-							-	F⁻	JC411, Clark (1963)
JC3435									-	-		-	-						-								Double Hfr, see JC182	JC182
JC4121		-		-					-			-					-	-	-					-			F⁻	JC1569
AB1206				-	-		-		-										-						-		Primary F'(F14) O, ilv, metB,...F	Pittard and Ramakrishnan (1964)
AT2699									-				-				-	-	-						-		F⁻	Taylor and Trotter (1972)
DG88	-	-		-		-	-	-	-		-								-		-		-		-		F⁻	Wolf, et al. (1968)
DG90	-	-		-		-	-	-	-		-							-	-		-				-		F⁻	"
DG111				-	-	-	-		-						-				-	-	-		-		-		F⁻	"
JF4		-										-						-	-							-	F⁻	JC3419
JF131		+/-										-						-	-							+/-	Secondary F'(F390), O, leu, pyrB, ...F	This paper
JF132		-										-						+/-	+/-							+/-	Secondary F'(F391), O, argG, pyrB, ...F	"
JF133		-										-						+/-	+/-							+/-	Secondary F'(F392), O, argG, pyrB, ...F	"
JC3368		-								-		-						+/-	-					-		-	Secondary F'(F50), O, argG, metC,...F	"
JF127		-										-						-	-							-	F⁻	JF4

* Blank spaces in the table imply that the gene is wild type. Symbols are those employed by TAYLOR and TROTTER (1972).

RESULTS

Isolation of F's from the double male: Hfr strains are by definition clonal populations of mostly Hfr cells in which the F plasmid is integrated into the chromosome. Hfr strains also contain variable numbers of F+ and F′ cells. The F′ cells carry two chromosomes, one of which, the F′ plasmid, consists of all or part of the F plasmid to which is covalently attached various lengths of the chromosome. The chromosome component of the F′ plasmid is detectable if the F′ carries Hfr markers normally transferred proximally (Low 1968), terminally (JACOB and ADELBERG 1959; BERG and CURTISS 1967) or both (SCAIFE and PEKHOV 1964; BRODA, BECKWITH and SCAIFE 1964; SCAIFE 1966).

The detection of both early and late markers on the same F′ plasmid isolated from an Hfr indicates close linkage of both these markers with the integrated F plasmid. This fact allows us to establish the presence of cells of the double male that possess one or two chromosomes for their genomes. If an F-*argG* plasmid is formed in the double male which possesses a single linkage group, then there is a possibility that the marker *metC* will be included on that F plasmid (Figure 1). On the other hand, if the genome of the double male consists of two linkage groups (Figure 1), then rather than the *metC* marker being included on the F plasmid the marker *pyrB* may be included. The probability that any terminal marker will be included on an F plasmid carrying a proximal marker is less than one; consequently only a fraction of any F-*argG* plasmids recovered will carry either *metC* or *pyrB*. The same argument can be made for the markers *leu* and *pyrB* or *leu* and *metC*.

Table 2 displays the results of crosses between JC182 and two F⁻ *recA*⁻ strains to detect and recover strains carrying F-prime elements generated by the double male. The table shows the recombination frequency (per 100 donor cells) for each class of recombinant, the number of putative F-prime donors, and the num-

TABLE 2

Characterization of F-prime strains sired by the double male, JC182

Recombinant class	Recombination frequency* JC1569	JF4	F-prime donors tested†	ArgG+ MetC+‡	Donor classes Leu+ PyrB+§	ArgG+ PyrB+¶	Leu+ MetC+‖
Arg+ {Sm^R}	0.003	0.007	12 (250)	1(JC3368)	0	0	0
Leu+ {Sm^R}	0.002	0.006	33 (500)	0	0	0	0
Ura+ {Sm^R}		0.005	7 (44)	0	1(JF131)	2(JG132, JF133)	0

* Recombinants per 100 input donor cells.

† Donors of selected marker to RecA⁻Spc^R, either JF127 or JC4121 (total number of transconjugants scored for donor ability).

‡ Ability of *argG*+ F-prime donors to sire ArgG+ MetC+ {Leu+} transconjugants with AT2699.

§ Ability of *pyrB*+ or *leu*+ F-prime donors to sire Leu+ Ura+ {Spc^R} transconjugants with JF127.

¶ Ability of *argG*+ or *pyrB*+ F-prime donors to sire ArgG+ PyrB {Spc^R} transconjugants with JF127.

‖ Ability of *leu*+ F-prime donors to sire MetC+ {Leu+ (JC1569) or Ura+ (JF4)} transconjugants with AT2699.

TABLE 3

Transmission characteristics of F-prime strains sired by the double male

Strain	F-prime markers	Primary selection	Unselected markers ArgG+	Sm^R	PyrB+	Leu+	MetC+	Recipient strain
JC3368	*argG+ metC+*	ArgG+ {Sm^R}	100	100	0	0	89	AT2699
JF131	*leu+ pyrB+*	Ura+ (PyrB+) {Sm^R}	0	100	100	100	0	JF127
JF132	*argG+ pyrB+*	ArgG+ {Sm^R}	100	0	24	0	0	JF127
JF133	*argG+ pyrB+*	Ura+ (PyrB+) {Sm^R}	68	0	100	0	0	JF127
		ArgG+ {Sm^R}	100	0	2	0	0	JF127

ber and type of each F-plasmid carrying two of the four markers examined. Putative F-prime donors were identified by their ability to transfer the selected marker to either JC4121 or JF127 using spectinomycin contraselection. Both of these spectinomycin-resistant strains are *recA*$^-$, and thus transconjugants for the selected markers should arise not through recombination but by inheritance of an F plasmid (Low 1968; GUYER and CLARK, unpublished results).

It was possible to test the 12 Arg$^+$ F-plasmid-carrying strains for donor ability of *metC*$^+$ by crossing them with AT2699. Arg$^+$ {Leu$^+$} transconjugants of such crosses were tested for the inheritance of Met$^+$ as an unselected marker (Table 3). One of the twelve (JC3368) formed Met$^+$ Arg$^+$ transconjugants (89% of Arg$^+$ were Met$^+$).

To test further the nature of the F′ plasmid in the strain JC3368, the strain was grown in the presence of acridine orange (10 μg/ml). After growth for 48 hours, 99% of the survivors were Arg$^-$ and had lost the capacity to transfer *argG*$^+$ or *metC*$^+$ to AT2699, while growth without acridine orange yielded only 1% Arg$^-$ nondonor cells. We conclude that the strain JC3368 carries an F-plasmid to which are linked *argG* and *metC*. This plasmid, carried by strain JC3368, is designated F50.

Among the seven Ura$^+$ {Sm^R} conjugants of JC182 and JF4 one, JF131, is able to sire Leu$^+$ Ura {Spc^R} progeny in crosses with JF 127. The frequency of inheritance of PyrB$^+$ among Leu$^+${Spc^R} transconjugants in the cross JF131 with JF127 is 100% (Table 3). Attempts to cure this F-plasmid (F390) from the host, JF131, have been unsuccessful. From an analysis of other F′ derivatives of the strain JF4, parent of JF131, it appears that JF4 is acridine orange-resistant.

Two other F′ strains have been recovered from crosses between the double male and JF4 (Table 2). Both are Ura$^+$ {Sm^R} conjugants and both donate *argG*$^+$ and *pyrB*$^+$ to JF127. The F-plasmids (F391, F392) in these two strains (JF132, JF133, respectively) conform to the F20 plasmid described by CLARK, MAAS and Low (1969). These two strains lose either Arg$^+$ or Pyr$^+$ spontaneously and both are streptomycin-sensitive, though streptomycin was used for counterselection. Possibly their survival is due to the streptomycin-resistant phenotype of the recipient strain. This is the expected behavior of a strain carrying the long F20 plasmid diploid for the streptomycin locus (*str*$^-$/*str*$^+$). Finally the decrease in linkage between *argG* and *pyrB* in the cross of JF133 and JF127 suggests the behavior of a long unstable F′.

Thirty-three F'*leu*$^+$-carrying strains were examined; none was found which carried *leu* and *metC*, though as described above, one carrying *leu* and *pyrB* was recovered.

DNA replication in the double male strain of Escherichia coli: In order to ascertain the proportion of cells of the double male in the one- or two-chromosome configuration, the temporal replication sequence of genetic markers located about the chromosmoe of *E. coli* was followed using the technique of WOLF, NEWMAN and GLASER (1968).

Assuming an exponential population of cells, the marker frequencies for different markers plotted against the map position of each marker describes a smooth curve (WOLF, NEWMAN and GLASER 1968; SUEOKA 1971). MASTERS and BRODA (1971) and BIRD *et al.* (1972) have presented evidence demonstrating that in exponential cultures of their strains of *E. coli* K12, the temporal replication sequence of chromosomal markers can best be described assuming a bidirectional replication from a single origin. Accordingly, an exponential culture of the double male was infected with bacteriophage P1 *vira* and after allowing for phage growth, bacteriophage was recovered. The transductant frequencies for different markers among total transductants from the double male lysate plotted against their map positions on the chromosome are shown in Figure 2. The results can be interpreted as evidence demonstrating bidirectional replication in the double male with an origin between *aroB* and *argG* and a terminus near *gal*. This result is in agreement with the data of MASTERS and BRODA (1971). The same pattern of marker replication was noted in the *recA*$^-$ derivative of the double male.

To examine further the nature of chromosome replication in the double male and thereby infer the structure of its genome, the pattern of transduction frequency for different markers was examined in cultures starved 90 min for two required amino acids (isoleucine and valine) in the presence of a heavy density label for DNA (5-bromouracil). Next, cells were infected with P1 *vira* in fresh complex medium without 5BU, and after lysis phage were recovered. Upon removal of the required amino acids, chromosome replication will continue in all cells until each finishes a round of DNA replication (LARK, REPKO and HOFFMAN 1963). Upon reaching the end, there will presumably be no reinitiation because this step is blocked by amino acid starvation, and DNA synthesis will halt (LARK, REPKO and HOFFMAN 1963). Starvation in the presence of a DNA density label (5-bromouracil) will therefore result in preferential 5BU incorporation in the terminal regions of the chromosomes.

For the purpose of assaying the labeled markers P1 carrying hybrid (5BU-light) DNA in phage was recovered by CsCl equilibrium sedimentation and used for transduction of the recipient strains. We expect markers near the terminal regions of the chromosome to contain more 5BU than those at the origin. In both the double male derivative, JC3327, and the *recA*$^-$ double male strain a 30% increase in DNA was noted during the starvation period; no heavy (5BU-5BU) labelled DNA was detected by CsCl equilibrium gradient centrifugation.

The experimental results displayed in Figure 3 show the pattern of the fre-

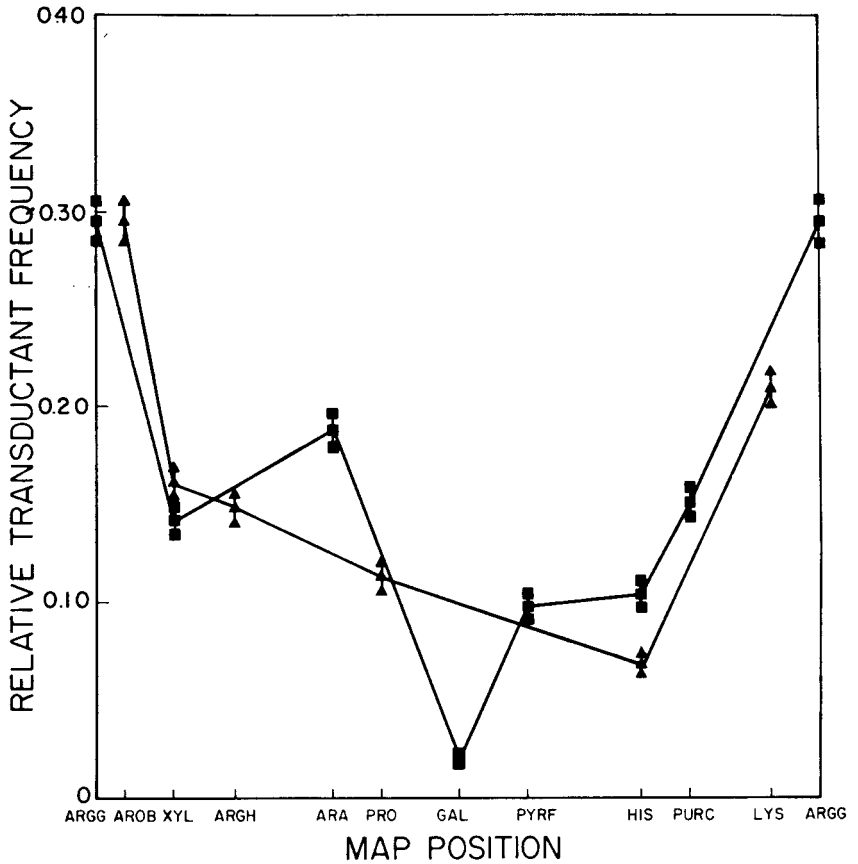

FIGURE 2.—Results of transductional marker frequency analysis of P1 *vira* lysate from an exponential culture of JC3327, a derivative of the double male. The two different transductional recipients are DG90 (■) and DG111 (▲). The frequency of each type of transductant as a proportion of the total number of transductants from a sample of the lysate grown on the exponential culture is plotted against the map position of the individual marker. The three data points represent the average and standard deviations of each determination. Lines are drawn to help the eye observe the patterns obtained with each recipient.

quency of each transductant class among total transductants of recipient cells infected with phage carrying hybrid DNA. This pattern is similar to that for the exponential population. A similar pattern was observed for the *recA*$^-$ derivative of the double male. In light of the discussion above, this result was unexpected since we expected to find the labelled phage to be enriched for markers near *gal*. Among possible explanations for the results are the following: (1) 5-bromouracil was incorporated randomly throughout the genome at the beginning of the starvation period; (2) 5-bromouracil was incorporated by repair replication throughout the chromosome; (3) amino acid starvation did not prevent initiation of DNA replication in the strains we used; or (4) growth under the conditions imposed by the experiment caused aberrant replication. We have not tested any of these possibilities.

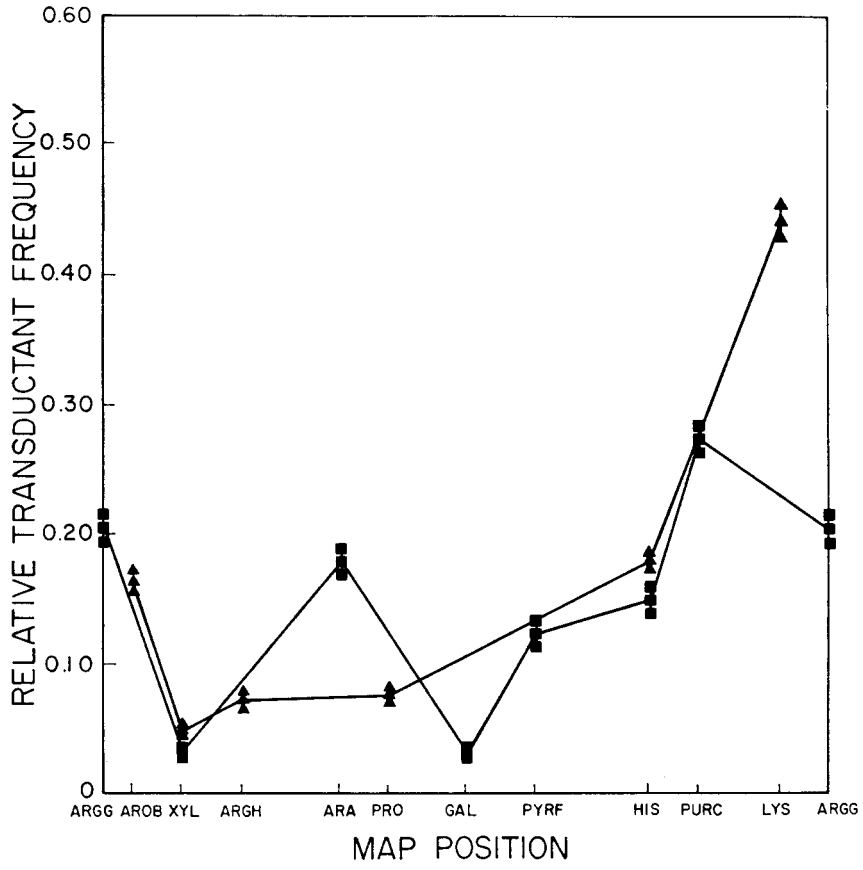

FIGURE 3.—Results of transductional frequency analysis of P1 *vira* lysate from a culture of JC3327, double male derivative, starved for required amino acids in the presence of 5-bromouracil. The two different transductional recipients are DG90 (■) and DG111 (▲). The frequency of each type of transductant as a proportion of the total number of transductants from a sample of the lysate grown on the starved culture is plotted against the map position of the individual marker. An average and standard deviation are plotted for each determination. The lines are explained in the legend of Figure 2.

One other source of error was investigated: The possibility that the technique does no measure accurately the number of gene copies in a population. To answer this question 10 Ara$^+$ transductants of DG90 from the experiment in Figure 3 were grown and P1 lysates made from these strains. The *ara* marker was chosen because of the high relative values for its transduction frequency. Each lysate was used to produce Leu$^+$ transductants of a Leu$^-$, Ara$^+$ strain. Three of the ten lysates formed significant numbers of Leu$^+$, Ara$^-$ clones. This suggests that in the sample chosen these three recombinants of DG90 represented suppressed *ara*$^-$ (hence Ara$^+$) clones, and the value for the transductant frequency of *ara* has been overestimated. Inasmuch as some suppressor loci are near the presumed terminus of replication (TAYLOR and TROTTER 1972), their contribution to P1 transducing particles may be higher in the terminus labelling experiment than

TABLE 4

Effect of growth in acridine orange (AO) up survival and donor ability of JC182 and parents

Strain	Survivors+AO* / Survivors—AO	Percent donors among survivors† —AO	+AO
A. Parents of JC182			
JC12	79%	100	100
JC158	75%	100	100
JC158F+	96%	100	0
B. Primary F-prime strain			
AB1206	0.6%	100	100
C. Double male			
JC182	58%	100	100
JC4112(recA-JC182)	34%	100	100

* Acridine orange 25 μg/ml in L-broth adjusted to pH 7.8 with 0.1 M NaOH.

† To test for donors the following crosses and selections were employed. For JC12, JC182 and JC411, Arg+ {Spc^R} recombinants were selected with JC4120. For JC158, JC158F+, JC182 and JC4112, Leu+ {Spc^R} recombinants were selected with JC4120. For AB1206 Met+ {Spc^R} recombinants were selected with JC4121.

in the marker frequency experiment performed with exponentially growing cells.

Acridine orange curing: If a portion of the double male cells consist of a genome with two separate units, one might be under control of the F-replicator. Growth in the presence of acridine orange would cure the double male strain of any DNA replicated autonomously by the F-replicator, and since the strain is haploid this loss would be lethal. Thus the behavior of the double male grown in the presence of acridine orange would deviate from its parents and conform to the behavior of a primary F′ strain, AB1206 (PITTARD and RAMAKRISHNAN 1964). Cells of JC182, JC12, JC158, and AB1206 were grown in LB containing 25 μg/ml acridine orange. At this concentration, viability and donor capacity of JC182, JC12 and JC158 were unaffected, whereas only 1% of the cells of AB1206 survived (Table 4). Survivors of acridine orange growth of the double male still exhibited the dual mode of chromosome transmission. As a control an F+ derivative of JC158 (one parent of JC182) was tested for curing. As expected, growth in AO-containing medium resulted in loss of donor capacity of the F+ derivative of JC158. Since the F50 plasmid can be cured in strain JC3368, neither parental sex factor in the double male is resistant to acridine orange-induced curing. It is of interest to note that among survivors of the primary F′ strain, AB1206, those tested were still primary F′ donors, not Hfr cells. This is in contrast to data reported by BERG and CURTISS (1967) and by SCAIFE (1966).

DISCUSSION

Evidence presented in this paper indicates that some of the cells of the double male strain exist in the single linkage group configuration. This has been shown by the recovery of two F′ elements: F-*argG metC* and F-*leu pyrB*. Only in the one chromosome configuration could these markers be linked. The possibility that we have not examined enough F′ plasmids sired by the double male and have

thereby failed to recover the rare *leu metC* F′ plasmid cannot be ruled out. On the basis of the acridine orange curing data (Table 4) the fraction of cells in the two linkage group configuration is less than one percent. This is based on the supposition that if there are two linkage groups, one is under replicative control of the F-replicator and is acridine orange-sensitive. Of 100 survivors of the double male grown in acridine orange containing medium all 100 retained the dual mode of chromosome gene transmission.

Data from the temporal replication sequence of genetic markers in exponentially growing cultures of the double male confirm data reported elsewhere (MASTERS and BRODA 1971). The weaknesses of the 5BU labelling technique reviewed above and the inherent problem of suppressor mapping by P1 transduction prevent any final conclusions from being drawn from those experiments.

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