SOME CHARACTERISTICS OF THE RESISTANCE TRANSFER FACTOR (RTF) EPISOME AS DETERMINED BY INACTIVATION WITH TRITIUM, P^{32}, AND GAMMA RADIATION

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ABSTRACT The resistance transfer factor (RTF) episome was studied by measuring its inactivation by Co^{60} gamma radiation, by incorporated P^{32}, and by tritium incorporated as tritium-labeled thymine. The D_{37} for Co^{60} irradiation was 7 to 9 $\times$ 10^4 rad. Growth of the bacteria harboring the RTF in BUdR (bromouracil deoxyriboside) increased the sensitivity of the RTF to the gamma radiation. The RTF was markedly inactivated by tritium after growth of the host (thymine requiring) bacteria in tritium-labeled thymine, thus further establishing the presence of thymine in the genome of the RTF. Assuming the efficiency of inactivation by P^{32} to be 10%, the phosphorus content of the RTF was estimated to be about 2 $\times$ 10^5 P atoms/episome. The data suggest the RTF contains double stranded DNA with a molecular weight of the order of 3 to 8 $\times$ 10^7.

INTRODUCTION

The resistance transfer factor (RTF) episome has been extensively studied by several investigators (Watanabe, 1963) but many of its physical characteristics are unknown. The analysis of satellite bands of DNA in *Proteus* infected with RTF has suggested it is a DNA-containing structure with a molecular weight of about 7% of that of the chromosome of *Proteus* (Falkow et al., 1963). To learn more about the RTF, a radiation inactivation study was initiated. The results have shown that RTF episomes transferred from cells grown in high specific activity H^3-thymine to nonradioactive recipients are inactivated by the tritium thus establishing that RTF contains thymine and thus, presumably DNA. The kinetics of inactivation of the RTF by gamma radiation are very similar to those of some double stranded DNA bacteriophages and yield a D_{37} identical with that of the λ phage of *Escherichia coli*. Inactivation of RTF by P^{32} yields data indicating that there are about 2 $\times$ 10^5

P atoms/episome. These data all suggest the RTF to be double stranded DNA-containing structure with molecular weight of the order of λ phage.

MATERIALS AND METHODS

Cultures. All experiments were done with strains of *Escherichia coli.* Donors were either a $K_{12}F^-$ requiring methionine (met^-) carrying the RTF factor (with information for resistance to chloramphenicol, 50 μg/ml; streptomycin, 25 μg/ml; and sulfatihazole, 200 μg/ml) or *E. coli* B3 requiring thymine or thymidine and carrying the same factor. The recipient was a $K_{12}F^-$ requiring arginine (arg^-).

Media. Penassay broth (Difco Laboratories Inc., Detroit) was used for all liquid cultures. Minimal medium (Davis and Mingioli, 1950) supplemented with the necessary amino acid was routinely used to measure population density of the auxotrophic *E. coli* cultures. Chloramphenicol (Parke, Davis and Co., Detroit) was without exception used at a concentration of 25 μg/ml.

Irradiations. Irradiations were accomplished with Co^{60} in a Model 200 Gammacell (Atomic Energy Commission of Canada, Ltd.) at a dose rate of 7.0 to 7.5×10^3 rad/min, with $K_{12}F^-met^-$ RTF or B3 RTF suspended in serum-containing penassay broth. The irradiations were performed in such a manner that the tubes to be most heavily irradiated were placed in the gamma cell chamber first and others added serially so that the irradiation of all samples ended at the same time. Immediately after exposure, the samples were placed in an ice bath to prevent cell multiplication.

Assay of Surviving Donor Bacteria. Surviving donor bacteria were scored on minimal medium containing methionine (for $K_{12}met^-$) or thymidine (for B3).

Transfer of the RTF Factor. One ml of the irradiated doner was mixed with 4 ml of *E. coli* $K_{12}F^-arg^-$ (2×10^9/ml). The conjugating mixture was statically incubated at 37°C. After 3 or 4 hr and at 24 hr, 2 ml samples were removed, centrifuged, and washed twice in saline. Appropriate aliquots were plated on minimal medium containing arginine and chloramphenicol or streptomycin. In early experiments streptomycin (25 μg/ml) was added to duplicate arginine agar plates and streptomycin and chloramphenicol to others. However, such plates always showed the same (within plating error) number of colonies as with arginine plus chloramphenicol. This result showed that the irradiation did not selectively inactivate either of the two markers and this practice was discontinued.

Tritium Inactivation. *E. coli* B3 RTF was grown in minimal medium containing 4 μg/ml H^3-thymine (6.6 c/mM, New England Nuclear Corporation, Boston). After growth to about 1×10^9 cells/ml, the bacteria were washed twice and incubated statically with *E. coli* $K_{12}arg^-$ at 37°C for 2 hr. Samples were removed for zero time controls and the mixture divided into aliquots half of which were stored at 4°C and the remainder at −196°C in liquid nitrogen. Samples were removed at regular intervals, thawed, and plated on appropriate media to determine the viability of donor cells, recipient cells with chloramphenicol resistance, and total recipient cells. Controls consisting of conjugated mixtures of similarly handled, but nonradioactive *E. coli* B3 RTF and $K_{12}arg^-$ recipients were also checked for viability at the same time to determine the effects of storage and freeze thawing in the absence of tritium.

Radioactive Phosphorus (P^{32}) Inactivation. *E. Coli* $K_{12}F^-met^-$ RTF was grown for about 7 generations in the presence of a medium prepared according to the method of Driskell-Zamenhof and Adelberg (1963) such that the final specific activity of P^{32} in

it was 64 mc/mg phosphorus. These cells were washed twice and incubated statically in pensassay broth with 10^9/ml *E. coli* $K_{12}arg^-$ as recipients. After 1 hr of conjugation, the mixtures were chilled in an ice bath and stored in the refrigerator. Samples were removed then and each day for the next 8 days, and the viability of donor cells, recipient cells resistant to streptomycin, and the total recipient cells, was determined by plating on methionine containing agar, arginine and streptomycin containing agar, and arginine containing agar, respectively. The effect of storage alone was determined by carrying cultures that were identical, with the exception that they contained no radioactivity, through the same procedures.

Radioactivity Determinations. All analyses for radioactivity were done with a Packard Tri-carb Spectrometer (Packard Instrument Co., La Grange, Illinois) using the liquid scintillation technique described earlier (Painter and Rasmussen, 1964). The absolute tritium content of bacteria grown in H^3-thymine was determined by extraction of an aliquot of washed cells with cold 4% perchloric acid (PCA) followed by alkaline hydrolysis and exhaustive extraction of DNA with hot (60°C) PCA. More than 90% of the cold PCA insoluble tritium was in the DNA fraction.

RESULTS

Inactivation by Gamma Radiation. The result of an experiment typical of several in which the episome-containing *E. coli* K_{12} was irradiated while suspended in serum-containing penassay broth is shown in Fig. 1. The donor inactivation curve

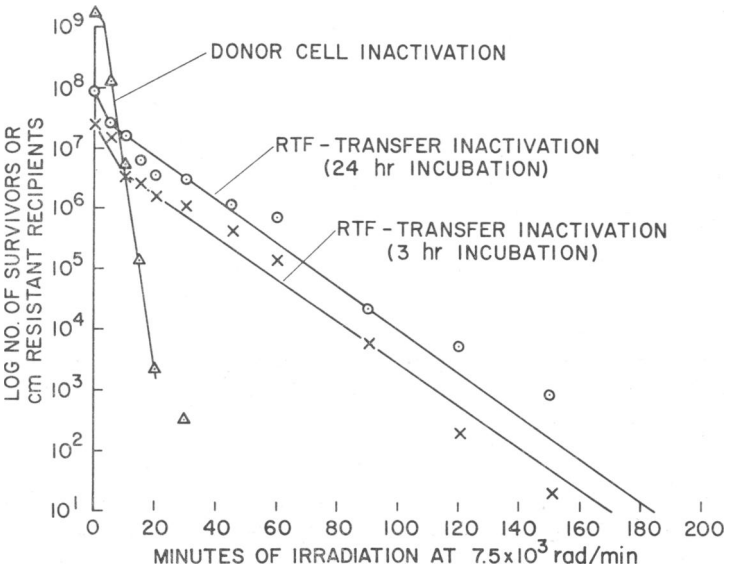

FIGURE 1 Inactivation of episome donors and of episomic transfer by Co^{60} gamma irradiation of RTF-infected *E. coli*, $K_{12}F^-met^-$ RTF suspended in serum-containing penassay broth. △ —— △, viable count of donor cells as determined by plating on methionine-containing minimal medium. × —— ×, count of RTF-containing recipient cells, $K_{12}F^-arg^-$, as determined on arginine- and chloramphenicol-containing minimal medium, after 3 hr incubation with donors. ⊙ —— ⊙, count of recipient cells after 24 hr incubation with donors.

shows a shoulder followed by essentially exponential inactivation. The dose required to reduce the population of viable donor cells to 37% of some other point on the straight line portion of the curve (D_{37}) averaged about 9×10^3 rad. The curve depicting the ability of the donors to transfer the RTF (the recipient curve) is a two component one, with a very abrupt break occuring at low doses. The results with 3 and 24 hr conjugation show identical inactivation kinetics, with the zero dose value difference accounting for the shifting of 24 hr data to higher values. At high doses many donor cells that had lost their reproductive integrity were still capable of transferring episomes to recipients. The D_{37} of the first component is very low. Fig. 2 shows the results of an experiment with points taken at low doses of gamma radiation. In this experiment the first component of inactivation was

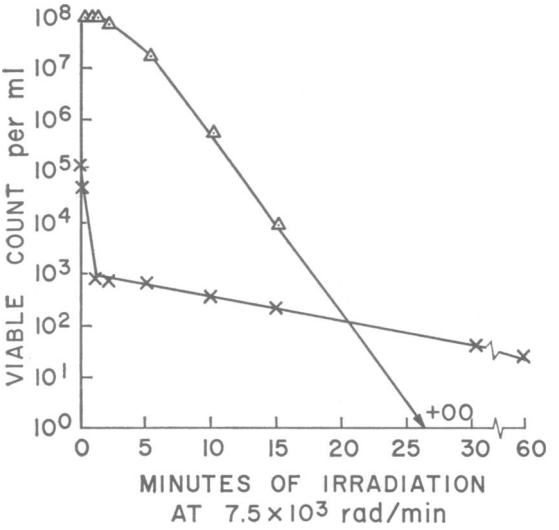

FIGURE 2 Inactivation of episome donors and of episomic transfer by relatively low doses of Co^{60} gamma irradiation. △ —— △, viable count of donor cells; × ——×, viable count of recipent RTF-containing cells after 3 hr incubation with donors.

completed at about 1 min of irradiation and its D_{37} is about 5×10^3 rad. This figure also demonstrates the shoulder of the donor inactivation curve much more clearly. The D_{37} of the second component of the inactivation of the episomal transfer (Fig. 1) is about 9×10^4 rad.

To determine if the inactivation curve of episome transfer really reflected inactivation of the episomic particle itself, the radiation sensitizing property of bromouracil deoxyriboside (BUdR) was utilized. It is well known that cells containing BUdR in their DNA are more sensitive to ionizing irradiation than are normal cells (Greer and Zamenhof, 1957). Therefore, *E. coli* B3 which requires thymine or thymidine for growth was infected with the RTF by mating with K_{12} RTF and the B3 RTF strain purified by growth in medium containing thymidine and chloramphenicol. These bacteria were then grown in a medium containing 4 μg/ml BUdR and 4 μg/ml thymidine. Prior studies had shown high incorporation of C^{14}-BUdR into the

DNA under these conditions. Other cultures were grown in the presence of thymidine only (8 μg/ml) and the two kinds of cultures were used as episome donors in the same way as previously described for broth cultures of *E. coli* K_{12}met$^-$ RTF. Results are shown in Fig. 3. The presence of incorporated BUdR enhances the radiation inactivation of the donor cell and of episomic transfer. A plot of the RTF transfer inactivation of B3 does not yield a two component curve as with the K_{12} strain, but the D_{37} of normally grown RTF is almost identical with that of the second component of the analogous curve with the K_{12} strain. The effect of BUdR in the bacterial DNA is such that the sensitivity to Co^{60} irradiation is increased by about 2.8 times, while the effect on episomal transfer inactivation is to increase its sensitivity by about 1.8 times.

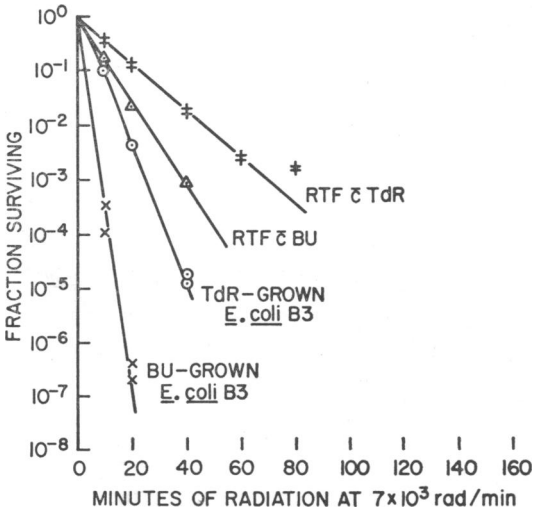

FIGURE 3 Inactivation of episome donors and of episomic transfer by Co^{60} irradiation of *E. coli* B3 RTF, with and without BUdR substitution in the DNA. ⊙ —— ⊙, viable count of thymidine-grown *E. coli* B3 RTF donor cells; × —— ×, viable count of BUdR-grown *E. coli* B3 RTF donor cells; + —— +, viable count of $K_{12}F^-$arg$^-$ recipients of thymidine-grown RTF, on arginine chloramphenicol-containing medium; △ —— △, viable count of $K_{12}F^-$arg$^-$ recipients of BUdR-grown RTF, on arginine- and chloramphenicol-containing medium.

Inactivation by Incorporated Tritium. The effects of tritium on the episome are summarized in Figs. 4 and 5. At 4°C (Fig. 4) inactivation of the episome occurs with essentially single hit kinetics, with a slightly slower rate of inactivation occurring after the 24th day. The original rate of inactivation is about one sixth of that of the steep exponential portion of the inactivation of the donor cells. The shoulder in the inactivation curve of the donor cells is probably an artifact, caused by growth of the culture after the zero time point since tritium inactivation does not show a shoulder at −196°C (Fig. 5). Previous workers have

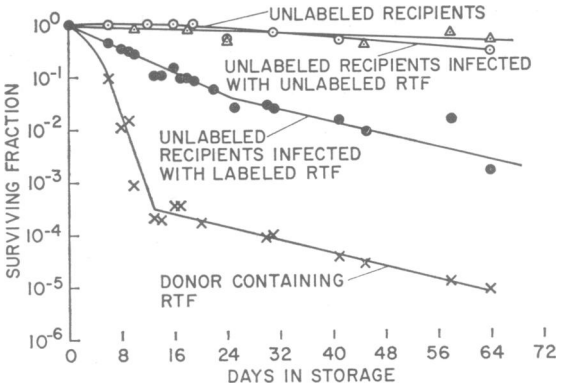

FIGURE 4 Inactivation of donor cells and of RTF by H^3-thymine in otherwise unlabeled recipient $K_{12}F^-arg^-$ cells, during storage at 4°C. × —— ×, viable count of donor *E. coli* B3 cells that had been grown in the presence of 6.7 c/mM H^3T; ● —— ●, count of H^3-RTF containing recipient cells as determined by plating on arginine- and chloramphenicol-containing agar; ⊙ —— ⊙, count of total recipient type cells as determined by plating on arginine agar without chloramphenicol; △ —— △, count of recipient $K_{12}F^-$ RTF cell that had been conjugated with nonradioactive donor *E. coli* B3 RTF.

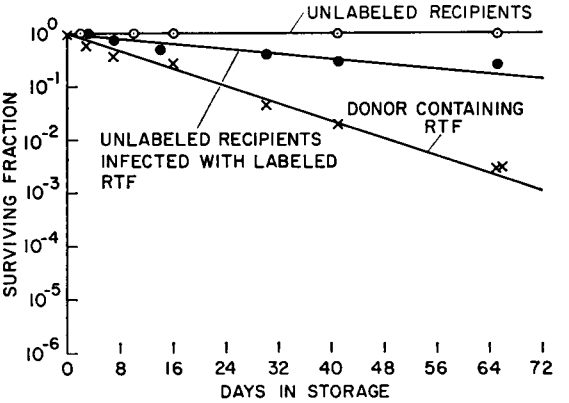

FIGURE 5 Similar to Fig. 4 except storage was at −196°C. Unlabeled recipients infected with unlabeled RTF curve not shown, but data are identical with the total unlabeled recipients.

found simple single hit kinetics for *E. coli* inactivation by tritiated thymidine (Person and Lewis, 1962; Apelgot and Latarjet, 1962). The exponential portion of the donor cell curve computes to a killing efficiency (α) of about 0.033, calculated according to the method of Person and Lewis (1962), and is in fairly good agreement with the results of Person (1963) for *E. coli* 15 T-mutants ($\alpha = 0.029$). Somewhat fewer than 0.1% of the cells are inactivated at a much lower rate; this is probably due to fewer tritium molecules in these cells, a condition that may have arisen by a small amount of cell division during the conjugation incubation.

At −196°C both the episomes and the donor cells show simple exponential inactivation kinetics. The slope of the curve for inactivation of the episome at −196° is about one fifth that at 4°, while for the donor cells, the slope at −196°C is about one ninth that of the exponential portion at 4°C.

Inactivation by Incorporated P^{32}. The inactivations of the episome and of the donor cells are shown in Fig. 6. A spontaneous inactivation of nonradioactive episomes occurred after the 3rd day of storage and the curve corrected for this

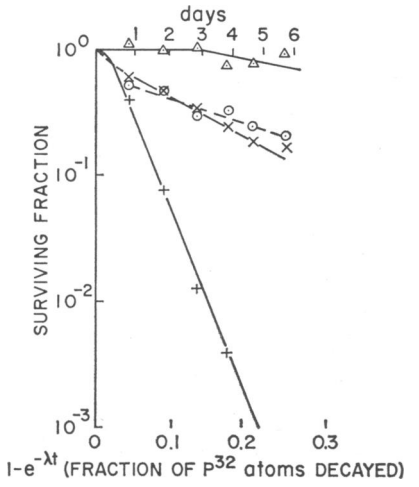

FIGURE 6 Inactivation of RTF and donor cells by incorporated P^{32}. + —— +, viable count of P^{32}-grown donor cells; × —— ×, viable count of recipient cells that had received the P^{32}-RTF, determined by plating on chloramphenicol agar; △ —— △, viable count of recipient cells that had received nonradioactive RTF; ⊙ —— ⊙, curve for survival of RTF, corrected for spontaneous decay of nonradioactive RTF.

loss is also plotted. A very short (<1 day) storage period apparently inactivated a relatively high proportion (15 to 35%) of the P^{32}-labeled episomes, but the remainder shows exponential inactivation for the next 5 days of storage.

The inactivation curve of the donor cells shows a small shoulder, and extrapolation of the exponential portion of the curve to zero dose yields an extrapolation number of 2. The exponential portion of the curve extends beyond 10^{-4} survival.

The inactivation by P^{32} can yield an estimate of the number of total phosphorus atoms, and thus of total molecular weight of the nucleic acid–containing unit, providing the inactivation efficiency is known. In the case of the RTF, this figure cannot be ascertained with certainty, but for all tested double standed DNA-containing biological units of viral size except one, the peculiar α phage of *Bacillus megatherium* (Cordes, Epstein, and Marmur, 1961), the killing efficiency (α) is in the order of 0.1 at 4°C (Stent and Fuerst, 1955). Using this value, an estimate of the number of phosphorus atoms per episome can be made using the equation of Hershey et al. (1951):

$$N = \frac{\log_{10} S}{-1.48 \times 10^{-6} \alpha A_0 (1 - e^{-\lambda t})}$$

where N is the total number of phosphorus atoms per particle, S is the fraction of surviving particles, α is the efficiency of killing per disintegration (here taken to be 0.1), A_0 is the original specific activity of P^{32}, and $1 - e^{-\lambda t}$ the fraction of P^{32} atoms decayed. If the straight line portion of the uncorrected data is used, $N = 3.55 \times 10^5$ P atoms/episome; with the data corrected for spontaneous decay of the episome, $N = 2.2 \times 10^5$ P atoms/episome.

When the same equation is used for the donor cells, taking 3×10^9 as the molecular weight of *E. coli* DNA (Cairns, 1963), α computes to 0.02, which is in excellent agreement with the figure determined by Fuerst and Stent (1956).

DISCUSSION

The gamma radiation inactivation scheme for episomic transfer with *E. coli* K_{12} RTF as donor exhibits a two component curve when plotted on semilog paper. The first component is steep and accounts for 50 to 99% of the inactivation. The break in the curve is abrupt and the second component is comparatively shallow. These results indicate that a relatively low dose of gamma radiation either inactivates the majority of the episomes or makes it impossible for the majority of the donor cells to transfer episomes. Falkow et al. (1963) have shown that DNA from RTF-infected *Proteus* exhibits two satellite peaks when analyzed by the equilibrium density gradient technique indicating that there may be two forms of RTF DNA in this cell. However, the D_{37} of the first component of the episome transfer curve is much less than that of the donor bacteria, and suggests a target even larger than the one(s) responsible for cell killing. Thus a form of the episome does not seem a likely target candidate and this component most probably represents inactivation of some cellular component involved in the transfer process. The fact that episomal donation does occur after heavy irradiation in a fraction of *E. coli* K_{12} cells and apparently in all *E. coli* B3 cells (since there is no evidence for this steep component in the inactivation curves for B3) shows that either some cells can accomplish donation in the absence of this factor, or that some cells contain the factor in a radioresistant form.

The ability of BUdR to so markedly sensitize episomal transfer inactivation kinetics in B3 establishes almost unequivocally that the action of the radiation is directly on the DNA of the RTF episome. Watanabe (1963) has shown that RTF transfer can occur within 1 min after mixing donor and recipient cells. Since this is an even shorter time than that required for the formation of inducible enzymes (Jacob and Monod, 1961), it appears that the components required for episomal transfer are already present in the cells before contact is made. Thus the action of BUdR must be directly on the episomal genetic material and not some consequence of damage to the host DNA. This concept is strengthened by the fact that the radiation sensitization by BUdR is different for cell killing than for episomal transfer. Furthermore, since the D_{37} of the normally (TdR)-grown B3 RTF transfer

inactivation curve is not statistically different from that of the second component of K_{12} RTF transfer inactivation curves, it is most probable that the latter is also due to inactivation of the episome itself. Thus it appears that the component of RTF transfer inactivation curves with D_{37} of 7 to 9 $\times$ 10^4 is almost certainly due to inactivation of the episome, per se.

The recent compilation of Kaplan and Moses (1964) suggests that with nucleic acid–containing biological units, a correlation exists between nucleic acid complexity (molecular weight, strandedness, and ploidy) and inactivation kinetics. In this context, it may be pertinent that the D_{37} of RTF falls in a range occupied only by double stranded DNA viruses (Group B of Kaplan and Moses). Indeed, the value of 7 to 9 $\times$ 10^4 rad is about the same as that given for phage λ.

The observations that growth of donor bacteria in H^3TdR can cause inactivation of RTF after transfer to nonradioactive recipients demonstrate clearly that the episome contains thymine in its genome. Since thymine is found only in DNA with the exception of a small amount in RNA that is no more than 1% of the uracil residues in *E. coli* (Littlefield and Dunn, 1958), this is strong presumptive evidence that the genetic material of RTF is DNA. This contention is further substantiated by the BUdR-gamma ray inactivation results, which, taken along with the density gradient results of Falkow et al. (1963), establishes the point almost beyond doubt.

The figure of 2.2 $\times$ 10^5 P atoms, computed for the RTF by assuming $\alpha = 0.1$, is almost identical to the 2.3 $\times$ 10^5 P atoms/phage, determined by Stent and Fuerst (1955) for λ phage. Along with the episomal D_{37} inactivation by gamma radiation, which also yields a value similar to that of λ phage, it seems plausible to assume that the RTF has a molecular weight close to that of this phage. If one takes α for P^{32} to be 1.0, as for single stranded phage, the molecular weight computes to a figure 4 to 5 times greater than that of known single stranded phages. Moreover, the D_{37} for gamma inactivation of the episome is about one fifth that of the single stranded phages. Thus, the information now available suggests the RTF to be an extrachromosomal genetic determinant, containing double stranded DNA of molecular weight 3 to 8 $\times$ 10^7.

It is tempting to speculate on the origin of this genetic element. We have shown that genetic exchange between the RTF and the host chromosome can occur in both directions (Ginoza and Painter, 1964). Thus it is conceivable that the episome arose by means of genetic recombinations, perhaps in a series of drug-resistant hosts, between the latters' DNA and the genome of a unit that originally was of (defective) bacteriophage origin. Somehow the competency to accomplish conjugation (or whatever is the physical basis of interbacterial RTF transfer) had to be attained, and this suggests some kind of interaction with the fertility (F) factor. A number of bacteriophage contain DNA of molecular weights that fall within the values estimated for the RTF, with the P1 phage a prime contender, due to its

ability to transduce the whole episome (Watanabe and Fukasawa, 1961). Experiments underway in this laboratory are aimed at elucidating more exactly the origin of this extrachromosomal genetic determinant.

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