The Number of Sex-Factors per Chromosome in *Escherichia coli*

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A substituted sex-factor of *Escherichia coli*, F'8 *gal*, was transferred to *Proteus mirabilis* by conjugation. The DNA of the episome was partially purified from *Proteus* DNA by preparative equilibrium centrifugation in caesium chloride, and by a bulk method using hydroxyapatite. The buoyant density of the episomal DNA is 1.707, corresponding to a (G+C) content of 47%. By optical renaturation the genetic complexity of the episomal DNA was found to be 76×10^6 daltons. RNA was synthesized *in vitro* by using the episomal DNA as template. By hybridizing this RNA with DNA extracted from *E. coli* carrying F'8 *gal*, it is shown that the number of copies of the episome per replicating chromosome is close to two during exponential growth. The episome makes up about 4.4% of the total DNA of the growing cells. The activities of galactokinase and galactose 1-phosphate uridylyl-transferase in cells with and without episomal and chromosomal *gal* genes were found to be proportional to the number of *gal* genes present, when the cells were induced with D-fucose, but not when they were induced with D-galactose.

The sex-factor F of *Escherichia coli* exists in the cell independently of the bacterial chromosome. A part of the chromosome may become associated with F, and can then be transferred with F by conjugation between different strains of *E. coli* (Jacob & Adelberg, 1959) and from *E. coli* to related bacteria such as *Proteus* (Falkow, Wohlheiter, Citarella & Baron, 1964) and *Serratia* (Falkow, Marmur, Carey, Spilman & Baron, 1961). The independent existence of the sex-factor means that extra copies of the genes associated with it are present in the cell. In principle, this should allow the study of gene-dosage effects. However, there is no general agreement about the time of replication of the sex-factor, and the number of sex-factors per cell.

In the present paper we describe a method for preparing large quantities of sex-factor DNA, based on the difference between its base composition and that of *Proteus* DNA. By using this DNA as a template for purified RNA polymerase complementary RNA was synthesized. By annealing the complementary RNA with DNA extracted from appropriate strains of *E. coli*, we were able to measure the number of sex-factors per replicating *E. coli* chromosome. This method should be useful in general for measuring the episome content of cells when the base compositions of cellular and episomal DNA is so similar that other methods are precluded.

METHODS

Bacterial strains. *E. coli* K12 strain E1 (*histr⁺str^R*) was derived by nitrosoguanidine mutagenesis from strain M2

(*histr⁺str^R*) (obtained from S. Brenner). Strain 9-3 (*histr⁺str^R Δgal*), which carries a deletion running from *att⁺* to beyond *aroG*, was derived from strain E1 by the method of Shapiro & Adhya (1969). The sex-factor F'8 *gal* (Hirota & Sneath, 1961) was introduced into these strains by F-duction. Strains NA9 (*tryhisstr^RgalE⁻*) (epimeraseless), NR3 (*tryhisstr^RgalK⁻*) (kinaseless) and TMG6 (*tryhisstr^RgalR⁻gal⁺*) were isolated after nitrosoguanidine treatment of strain E1. Strains 9S1 and 3S1 are *gal⁻* negative homogenotes isolated after infecting strains NA9 and NR3 with F'8. Strain PM1 of *Proteus mirabilis* (*lacgal⁺nic*) was obtained from Dr S. Falkow (Falkow *et al.* 1964).

Growth conditions. Strain PM1 (F'8) was grown at 37°C in a 14-litre New Brunswick fermentor in 3 × D salts (Fraser & Jerrel, 1953) supplemented with 0.1% casamino acids (casein hydrolysate), 0.2% galactose and 0.02% nicotinic acid and harvested in mid-exponential phase. Strains of *E. coli* were grown at 37°C with vigorous shaking in 3 × D salts supplemented with 3% casamino acids, 0.2% glycerol and 0.02% L-tryptophan and also harvested in mid-exponential phase. The generation time of the *E. coli* was close to 50 min in every case. Labelling of the DNA was achieved by adding 50 μCi of ¹⁴C-labelled uracil/litre three generations before harvesting.

DNA purification. (a) Large cultures of *Proteus*. Packed cells (20–40 g) were suspended in 400 ml of buffer NTE (0.1 M-NaCl, 10 mM-tris-HCl, 2 mM-EDTA, pH 7.5 at 0°C) and lysed by adding 0.05 vol. of 10% sodium lauryl sulphate and warming the suspension in a 45°C bath. Predigested pronase (30 min at 37°C) was added to a final concentration of 200 μg/ml and the solution was incubated at 37°C until it became substantially clear (2–4 h). It was then extracted twice with an equal volume of water-saturated phenol by gently swirling in a 2 litre flask (20 min) followed by centrifugation. The aqueous layer was dialysed at room temperature first against

3 changes of 10mM-tris-HCl buffer, pH 7.5, containing 0.3M-NaCl and then against buffer NTE. The solution was treated with pancreatic ribonuclease (50 µg/ml) for 3 h at 37°C followed by pronase (200 µg/ml) for 3 h at 37°C and extracted with phenol and dialysed as before. The ribonuclease-pronase-phenol treatment was repeated and after dialysis against 0.01M-tris-HCl buffer, pH 7.5, containing 0.3M-NaCl the DNA solution was finally dialysed exhaustively against 0.12M-sodium phosphate (equimolar, pH 6.8; see below).

(b) Small cultures. Small amounts of *Proteus* and *E. coli* were suspended in cold buffer NTE (4g/100ml) and treated with lysozyme (0.5 µg/ml) for 5 min in an ice-bath. Sodium lauryl sulphate was added to 1% and the flask was transferred to a 37°C bath. The cells usually lysed while the temperature was in the region of 10–15°C, and 200 µg of pronase/ml was then added. After 1 h at 37°C the solution was twice extracted with phenol and dialysed as described above. The dialysed solution was treated with 50 µg of ribonuclease/ml (1 h) and 200 µg of pronase/ml (1 h), extracted twice with phenol and dialysed. The ribonuclease-pronase-phenol procedure was then repeated twice more and the solution was finally dialysed against 1.5mM-sodium citrate buffer, pH 7, containing 15mM-NaCl (DSC). The DNA was then sedimented by 16 h centrifugation at 4°C and 30000 rev./min in an MSE 8×40 aluminium rotor and the pellets were gently dissolved in a small volume of DSC buffer.

All DNA solutions were checked for the presence of cold-acid insoluble and alkali-sensitive material. The DNA from the large cultures of *Proteus* contained about 15% of alkali-sensitive extinction (260nm). This material passed directly through the hydroxyapatite column. The DNA from the smaller cultures was used only if more than 95% of the total extinction (260nm) and radioactivity was alkali-resistant and cold-trichloroacetic acid-insoluble.

The specific radioactivities of the labelled DNA preparations were determined from their extinction at 260nm, taking 20 as the extinction of a 1 mg/ml solution, and from the radioactivity of 0.1 ml samples spotted on 3 cm membrane filters (see below) and counted in a liquid-scintillation counter.

Hydroxyapatite treatment. A column equipped with a water jacket and thermistor probe was used as described by Miyazawa & Thomas (1965). All phosphate buffers were dilutions of a 1.2M stock solution containing 0.6M-Na₂HPO₄ and 0.6M-NaH₂PO₄. The pH of this solution is about 6.4 and rises to 6.8 when diluted tenfold with water.

RNA preparation. ³²P-labelled *E. coli* ribosomal RNA was prepared as described by Bishop (1970). ³²P-labelled RNA (cRNA) was synthesized *in vitro* by using *Micrococcus* RNA polymerase (fraction VI described by Nakamoto, Fox & Weiss, 1964) and [α -³²P]ATP and purified as described (Bishop, 1969).

Purification of episomal DNA by isopycnic centrifugation. The DNA solution was adjusted to 40 µg/ml and 56% (w/w) CsCl and 12 ml portions were overlaid with liquid paraffin and centrifuged for 90 h at 35000 rev./min at 20°C in the MSE 8×25 aluminium rotor. Fractions were collected from the bottoms of the tubes and those immediately preceding the main band of DNA were pooled and dialysed against DSC buffer. This material was again adjusted to 56% (w/w) CsCl and centrifuged to near

equilibrium as before. The leading peak was collected and dialysed against DSC buffer.

Analytical isopycnic centrifugation was carried out at 44000 rev./min for 16 h at 25°C in 56% (w/w) CsCl.

Band sedimentation in alkali. Single-strand molecular weights were determined in 0.9M-NaCl-0.1M-NaOH by the method of Studier (1965).

Optical renaturation. The DNA was adjusted to 0.18M-NaCl-1mM-sodium phosphate buffer and 2.5 ml was brought to the renaturation temperature in the temperature-controlled housing of a Unicam SP.800 spectrophotometer. The DNA was denatured by adding 0.25 ml of 0.36M-NaOH. After 10 min the solution was neutralized by adding 0.25 ml of 0.24M-H₃PO₄ at 65°C and extinction recordings (260 nm) were begun immediately. At the end of the experiment the total hyperchromic extinction of the DNA was measured by bringing the sample to 95°C.

The second-order rate-constant of renaturation was calculated by a method similar to that of Wetmur & Davidson (1968). However, we define k_2 by $dD/dt = -k_2 D^2$ where D is the concentration of bases (mol/l) in denatured DNA. Hence:

$$1/D = k_2 t + 1/D_0 \quad (1)$$

where D_0 is the concentration of denatured bases at $t=0$. We let E_0 , E_∞ and E_t equal the extinction (260 nm) of the DNA at $t=0$, $t=\infty$ and $t=t$ respectively, and knowing E_0 , we calculate $E_\infty = 0.74 E_0$ (Wetmur & Davidson, 1968). We plot $1/(E_t - E_\infty)$ against t according to the relationship:

$$1/(E_t - E_\infty) = k'_2 t + 1/(E_0 - E_\infty) \quad (2)$$

and find the slope of the line, k'_2 . From eqns. (1) and (2):

$$k_2 = k'_2 \times 2380 \quad (3)$$

When the DNA contains two independently renaturing components (*Proteus* and λ or *Proteus* and F'8 in this case) the plot of $1/(E_t - E_\infty)$ against t is a curve that at long annealing times comes to approximate the slope of the more slowly renaturing component (Wetmur & Davidson, 1968). The early part of the curve approximates a straight line which is a complex function of the k'_2 values of the two components (see Wells & Birnstiel, 1969). In the present cases we know or can estimate the proportion of the total DNA which is made up by the slowly renaturing component, and consequently we can very easily discount the contribution it makes to the extinction of the fully denatured DNA. The extinction of the slower component changes due to renaturation, but in practice the change is slow enough, in the present case, that we can ignore it. If we let p equal the proportion of the total DNA made up by the rapidly renaturing component, we write: $1/[E_t - (1-p)E_0 - pE_\infty] = k'_2 t + 1/p(E_0 - E_\infty)$, or

$$1/[E_t - E_0(1 - 0.26p)] = k'_2 t + 1/0.26pE_0 \quad (4)$$

and find k'_2 from the plot of $[E_t - E_0(1 - 0.26p)]$ against t .

DNA-RNA hybridization. This was carried out by the membrane filter method (Gillespie & Spiegelman, 1965) as described by Bishop (1969). The annealing conditions were: cRNA, 2× SSC, 65°C, 16 h; ribosomal RNA, 2× SSC, 65°C, 5 h.

Enzyme assays. The bacteria were grown in glycerol-casamino acids medium at 37°C from an initial E_{650} 0.05

to E_{650} 0.6 with a generation time of 50–55 min and harvested by centrifugation. D-Fucose (5 mm) was added in certain cases at E_{650} 0.1. The cell pellet was washed by resuspension in cold 0.1 M-potassium phosphate buffer (pH 7)–10 mM-glutathione–1 mM-EDTA (magnesium salt) and recentrifuged, and was finally taken up in a volume of the same medium equal to 0.3 vol. of the harvested culture. The suspension was passed through a Sorvall French pressure cell at 20000 lb/in² at less than 8°C and cell debris was removed by centrifugation. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Galactokinase and galactose 1-phosphate uridylyltransferase activities were measured as described by Buttin (1963) with the following modifications. Glycylglycine was replaced by tris-HCl buffer of the same pH and concentration. The conversion of [¹⁴C]galactose into [¹⁴C]galactose 1-phosphate was measured by diluting the 0.05 ml reaction mixture 20-fold with water and then spotting 0.1 ml samples on to 2.5 cm Whatman DEAE-cellulose paper discs. These were thoroughly washed with water, dried and assayed in a liquid-scintillation counter. [¹⁴C]Galactose 1-phosphate and UDP-[¹⁴C]galactose were separated by first converting the galactose 1-phosphate into galactose. The 0.05 ml reaction mixtures were chilled by adding 0.7 ml of ice-cold water, placed in a boiling-water bath for 10 min and again chilled. Alkaline phosphatase (0.25 ml of 0.1 mg/ml solution in 0.4 M-tris-HCl buffer, pH 8 at 37°C) was added and the mixtures were incubated for 2 h at 37°C. Samples (0.1 ml) were spotted on to Whatman DEAE-paper discs, washed with water, dried and the radioactivity counted. Zero-time controls were run with all assays and subtracted from the experimental values. Specific activities are expressed as enzyme units/mg of protein, where the unit is defined as the amount of enzyme catalysing the synthesis of 1 μ mol of product/h.

RESULTS

Initial characterization of F'8. F'8 gal was transferred from *E. coli* to *P. mirabilis* (strain PM1) by conjugation. Colonies which segregated the *gal*⁻ phenotype at a low but significant rate were selected for further study (Falkow *et al.* 1964). DNA was extracted from *P. mirabilis* PM1 and *P. mirabilis* PM1 (F'8) and analysed by isopycnic centrifugation in caesium chloride. The results are shown in Fig. 1. *P. mirabilis* PM1 (F'8) showed a shoulder on the heavy (high G+C) side of the main band which was not seen in DNA from *P. mirabilis* PM1 itself, but there was no clear satellite peak. By contrast, DNA from *P. mirabilis* PM1 (F'1) showed a distinct satellite (Fig. 1). Genetic evidence shows that F'1 is larger than F'8.

DNA from *P. mirabilis* PM1 (F'8) was banded in a caesium chloride gradient and DNA was collected from the heavy side of the band. When analysed by isopycnic centrifugation in caesium chloride, this DNA showed two components (Fig. 2). One, with the same buoyant density as *P. mirabilis* PM1 DNA (1.698) is presumably a representative sample of *P. mirabilis* DNA. The other has a

buoyant density of 1.707, corresponding to a (G+C) content of 47%. No such component was found when similar samples of *P. mirabilis* PM1

Fig. 1. Isopycnic-density-gradient centrifugation of DNA from (a) strain PM1, (b) strain PM1(F'1) and (c) strain PM1 (F'8). The buoyant-density markers are *Micrococcus lysodeikticus* ($\rho=1.731$) and *Pseudomonas aeruginosa* ($\rho=1.736$) DNA.

Fig. 2. Isopycnic-density-gradient centrifugation of DNA from strain PM1 (F'8) enriched for the episomal DNA by preparative CsCl centrifugation. The buoyant-density marker is *Micrococcus* DNA ($\rho=1.731$).

Fig. 3. Optical melting profiles of *Proteus* (PM1, ○) and *E. coli* (M.R.E. 600, ×) DNA. (a) The DNA was kept at the temperatures shown for 10 min in 0.024M-sodium phosphate buffer, pH 6.8, and quickly chilled. Its extinction (260nm) was then measured at 40°C. (b) The DNA was progressively kept at each temperature for 10 min and its extinction measured directly.

DNA were analysed, and this is therefore taken to be the DNA of the episome F'8.

Bulk purification of F'8 DNA. The method depends on the fact that the (G+C) content of F'8 DNA is 47% whereas that of *P. mirabilis* is 40%. As a result, F'8 DNA becomes denatured ('melts') at a temperature 3.5°C higher than that at which *P. mirabilis* DNA 'melts' (Marmur & Doty, 1962). The method involves two steps. In the first, the DNA is subjected to conditions calculated to denature the bulk of the *P. mirabilis* DNA without greatly denaturing the F'8 DNA. The solution is then chilled, so that DNA that is only partially denatured will return to the original double-stranded condition. DNA strands that have separated because their denaturation is complete will be unable to do so (Geiduschek, 1962). As an illustration, Fig. 3(a) shows the respective tendencies of *P. mirabilis* DNA and *E. coli* DNA to return to the double-stranded state after controlled denaturation at different temperatures in 24mM-sodium phosphate buffer, pH 6.8. These profiles may be compared with the optical melting curves of the two types of DNA in the presence of the same concentration of salt (Fig. 3b). As reported by Geiduschek (1962) there is a difference of several degrees in the temperatures at which 50% of the DNA is found denatured by the two methods. From these experiments the temperature of 81°C was chosen for the controlled denaturation of DNA from *P. mirabilis* PM1 (F'8).

The partially denatured DNA was chilled, heated to 70°C, adjusted to 0.12M-sodium phosphate and immediately passed over a short column of hydroxy-

Fig. 4. Chromatography of unlabelled DNA from *Proteus* (PM1, —) and labelled DNA from *E. coli* (M.R.E. 600,).

apatite equilibrated with 0.12M-sodium phosphate at 75°C. The denatured DNA passed directly through the column and could be completely washed out with 0.12M-sodium phosphate (Fig. 4). The DNA adsorbed to the hydroxyapatite should consist of (a) F'8 DNA, (b) *P. mirabilis* DNA that was only partially denatured by the heat treatment, and resumed the double-stranded form when chilled and (c) cross-linked *P. mirabilis* DNA (Alberts & Doty, 1968), which renatured rapidly on chilling because of the cross-linkage. Fig. 4 shows an experiment in which a very small amount of ^{14}C -labelled *E. coli* DNA was added to unlabelled *P. mirabilis* DNA. The bulk of the absorbing material (*P. mirabilis* DNA) passes directly through the hydroxyapatite at 75°C and the bulk

Fig. 5. Rechromatography of unlabelled DNA from strain PM1 (F'8). The 0.3M-sodium phosphate buffer, pH6.8, eluate from three columns run like that shown in Fig. 4, with only strain PM1 (F'8) DNA, was pooled, dialysed against 0.12M-sodium phosphate buffer and rechromatographed.

Fig. 6. Optical melting profiles of *Proteus* DNA (—) and DNA enriched for F'8 by hydroxyapatite chromatography (----). The samples were kept at the temperatures shown for 10 min and their extinction measured at 260 nm.

of the radioactivity (*E. coli* DNA) is adsorbed. To remove the *P. mirabilis* DNA as far as possible, the hydroxyapatite column was heated to 91.5°C, at which temperature most *P. mirabilis* DNA 'melts' in 0.12M-sodium phosphate and elutes from the column (Fig. 4). The temperature was lowered to 75°C and remaining single-stranded DNA was removed by thorough washing with 0.12M-sodium phosphate. Double-stranded DNA was then eluted from the column with 0.3M-sodium phosphate. Fig. 4 shows that the radioactive *E. coli* DNA is eluted from the column with 0.3M-sodium phosphate. In most cases the DNA recovered from the column was thoroughly dialysed against 0.024M-

sodium phosphate and the fractionation procedure was repeated (Fig. 5). The DNA was then concentrated by centrifuging the solution at 35 000 rev./min in a Spinco no. 40 or MSE 10 × 10 Ti or 8 × 25 Ti rotor for 16 h at 2–5°C and dissolving the pelleted material in 0.1 × SSC.

Up to 20 mg of DNA could be treated in this way at a time, and the overall recovery was about 2% of DNA containing about equal amounts of *P. mirabilis* PM1 and F'8 DNA (see below). Assuming that the original PM1 (F'8) DNA contains 4% of F'8 DNA the yield is 25%.

Analysis of the product. The product of the hydroxyapatite fractionation was characterized in three ways.

(a) Melting profile. The melting profile of the DNA product is shown in Fig. 6 compared with the melting profile of *P. mirabilis* DNA. The profile of the DNA recovered from hydroxyapatite shows an inflexion point at about 76°C. The first part of the profile corresponds in melting temperature (T_m) with *P. mirabilis* PM1 DNA. The second part, with a T_m of 78.5°C, is assumed to be due to the F'8 DNA. The T_m of 78.5°C indicates a (G + C) content of 50%, which is rather higher than the value of 47% suggested by caesium chloride density-gradient centrifugation. This may be due to the base-compositional heterogeneity of F DNA (Falkow *et al.* 1961). The hyperchromicity of this DNA fraction is only 22%. This is taken to be due to the presence of single-stranded *P. mirabilis* DNA. Assuming that single-stranded DNA shows no decrease at 65°C from its extinction at 90°C in 0.024M-sodium phosphate, and that $E_{260(90)}/E_{260(65)} = 1.38$ for double-stranded DNA (see Fig 3b) the proportion of double-stranded DNA in the sample can be calculated with reasonable accuracy from $3.63 [1 - (E_{260(65)}/E_{260(90)})]$ and the proportion of F'8 DNA from $3.63 [1 - (E_{260(76)}/E_{260(90)})]$ where $E_{260(65)}$, $E_{260(76)}$ and $E_{260(90)}$ represent the extinction of the sample at 65°, 76° and 90°C respectively. In the example shown the proportion of double-stranded DNA is calculated to be 64%, and of F'8 DNA 39%.

(b) Optical renaturation analysis. The single-stranded molecular weight of the DNA was measured and the DNA was renatured in a solution containing 0.18g-equiv. of Na^+/l , at 65°C. Two components of renaturation are present: *Proteus* DNA with a complexity of about 2700×10^6 daltons (Table 1) and F'8 DNA with a much lower complexity. The existence of two components is clearly shown in Fig. 7.

To find the complexity of F'8 DNA it is necessary to obtain an accurate estimate of the second-order rate-constant of this component alone (Wetmur & Davidson, 1968). In the present case the relative amounts of F'8 DNA and *P. mirabilis* DNA can be

Table 1. *Renaturation results*

k_2 is the second-order rate constant of DNA renaturation, (mol of nucleotide). $l^{-1} \cdot s^{-1}$. M is the single-strand molecular weight. The term 'kinetic complexity' is defined by Wetmur & Davidson (1968). The expected values (last column) are literature values for the non-replicating DNA content. The observed values are calculated relative to the expected value for λ DNA in the complex system (line 1) by the method described in the text.

Type of experiment	Source of DNA	$10^6 \times$ Single-strand mol.wt.	Observed k_2 ($l \cdot mol^{-1} \cdot s^{-1}$)	$10 (k_2 / \sqrt{M})$	$10^{-8} \times$ Kinetic complexity	
					Observed	Expected
Complex (in the presence of <i>Proteus</i> DNA)	λ	0.25	11.5	230	—	0.31
	F'8 <i>gal</i>	0.44	6.2	94	0.76	—
Simple (no heterologous DNA)	λ	0.25	11.5	230	0.31	0.31
		0.34	13.4	223	0.32	
		3.20	48.0	266	0.27	
	<i>E. coli</i>	0.25	0.16	3.2	22.3	27
		0.28	0.175	3.3	21.6	
	<i>Proteus</i>	0.27	0.175	3.3	21.6	
		2.10	0.450	3.1	23

Fig. 7. Renaturation of a complex DNA preparation. A mixture containing equal parts of λ and *Proteus* DNA was prepared and sonicated briefly. The single-strand molecular weight was found to be 0.25×10^6 .

determined from the melting profile of the sample. This greatly simplifies the analysis of the renaturation data. In the early part of the renaturation period the change in extinction is mainly due to the less complex (faster renaturing) DNA component, F'8 in this case. Since the proportion of *P. mirabilis* DNA is known, its contribution to the extinction of the sample can be discounted, and k_2 for the F'8 DNA calculated by eqns. (4) and (3) (see the Methods section). The renaturation of the *P. mirabilis* DNA, which is proceeding at a slow rate, introduces some curvature into what would otherwise be a straight line (Fig. 8). However, the curvature is insignificant at the shortest annealing times. Fig. 8 shows two experiments carried out in this way. In one of these, a mixture of *P. mirabilis*

and λ DNA was annealed. The k_2 for λ DNA calculated from this experiment is in good agreement with the k_2 found by annealing λ DNA of the same size alone (Table 1). In the second experiment shown in Fig. 8, purified F'8 DNA containing contaminating *P. mirabilis* DNA was annealed in the same way. The kinetic complexity of F'8 DNA was estimated by comparison with λ DNA by using the relationship (Wetmur & Davidson, 1968):

$$\left(\frac{C_b}{C_a}\right) = \left(\frac{k_{2a}}{M_a^{\frac{1}{2}}}\right) \left(\frac{M_b^{\frac{1}{2}}}{k_{2b}}\right)$$

where C , k_2 and M are the kinetic complexity, rate constant of renaturation and single-strand molecular weight, respectively, of DNA types a and b. The value found, 76×10^6 daltons, is roughly what is expected for DNA containing F' (45×10^6 daltons; Freifelder, 1968) and about 1% of the *E. coli* chromosome (27×10^6 daltons). Table 1 also shows a control experiment in which λ DNA of different molecular lengths was renatured. As shown by Wetmur & Davidson (1968) the $k_2/M^{\frac{1}{2}}$ ratio is constant. Samples of *E. coli* DNA were also renatured. The kinetic complexity calculated as described above is in good agreement with the expected value (Table 1). The kinetic complexity of *Proteus* DNA was found to be indistinguishable from *E. coli* DNA, in agreement with the results of Kohne (1968) and Falkow, Haapala & Silver (1969). The absolute k_2 values for *E. coli* DNA shown in Table 1 are in reasonable agreement with those reported by Wetmur & Davidson (1968) when allowance is made (i) for single-stranded molecular weight, (ii) for the difference in salt concentration during renaturation and (iii) for the fact that we have defined k_2 by $dD/dt = -k_2 D^2$ (Britten & Kohne,

Fig. 8. Renaturation of λ and F'8 DNA in the presence of *Proteus* DNA. The renaturation of λ (a) or F'8 (b) DNA is shown after discounting the contribution of *Proteus* DNA to the total extinction. See the text for further details.

Fig. 9. Hybridization of cRNA made with F'8 DNA as a template with DNA from *E. coli* strains 9-3 (O) and 9-3 (F'8) (x).

Fig. 10. Hybridization of purified *E. coli* ribosomal RNA with DNA from *E. coli* strains 9-3 (O) and 9-3 (F'8) (x).

1966; Thrower & Peacocke, 1968) rather than by $dD/dt = -k_2(D/2)^2$ (Wetmur & Davidson, 1968).

(c) Positive identification as F'8. The experiments described below show that RNA transcribed *in vitro* from the purified DNA hybridizes to a much greater extent with DNA from *E. coli* strain 9-3 (F'8) than with DNA from strain 9-3 without the factor. This positively identifies F'8 as a major component of the purified DNA.

Measurement of the number of episomes per chromosome in E. coli. cRNA was transcribed *in vitro* from the partially purified F'8 DNA by using *Micrococcus* RNA polymerase (Nakamoto *et al.* 1964) with $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ as the labelled substrate.

^{14}C -labelled DNA was prepared from strains 9-3 and 9-3 (F'8 gal) of *E. coli*. Strain 9-3 has a deletion from the λ -attachment site to beyond *aro-G*, a region including all the genetic markers known to be carried by F'8 gal. The two types of DNA were annealed with the cRNA by the membrane-immobilization method (Gillespie & Spiegelman, 1965) with the results shown in Fig. 9. The cRNA hybridized to a much greater extent with strain 9-3 (F'8 gal) DNA than strain 9-3 DNA. As a control, membranes prepared at the same time were annealed with ^{32}P -labelled ribosomal RNA from *E. coli*. The two types of DNA hybridized with very similar amounts of ribosomal RNA (Fig. 10) showing that their capacity to hybridize with complementary

Fig. 11. Reciprocal plot of the hybridization of cRNA made with F'8 DNA as template, with DNA from *E. coli* strains 9-3 (O) and 9-3 (F'8) (x). ⊗, 9-3 (F'8)–9-3. For further details see the text.

RNA was the same. Thus the different amounts of hybridization found with cRNA must be due to the episomal DNA. This experiment proves that the DNA purified by means of hydroxyapatite contains F'8 *gal* DNA.

The experiment shown in Fig. 9 provides a means of measuring the number of episomal elements per cell in the exponentially growing culture of strain 9-3 (F'8). The saturation value for each type of DNA was determined as previously described (Bishop, 1969; Bishop, Robertson, Burns & Melli, 1969) (Fig. 11). The difference between the two values ($1.96 - 0.50 = 1.46\%$) gives the percentage of strain 9-3 (F'8 *gal*) DNA that will hybridize with saturating amounts of cRNA complementary to F'8 *gal*. A slightly different value (1.56%) is found if the differences between corresponding experimental values for strain 9-3 and strain 9-3 (F'8 *gal*) are separately measured and plotted (Fig. 11). It has been shown by using DNA and cRNA of three types (bacteriophages λ and T4 and *E. coli*) that in each case the extrapolated saturation value was close to $0.4 \mu\text{g}$ of RNA hybridized/ μg of DNA (Bishop, 1969). That is to say, only 40% of the complementary DNA present becomes hybridized with cRNA. Making the appropriate correction, we estimate

Fig. 12. Hybridization of cRNA synthesized by using F'8 DNA as the template. x, 9-3 (F'8)–9-3; O, 9-3 (with 1.6% F'8 DNA added)–9-3.

that strain 9-3 (F'8 *gal*) DNA contains 3.7–3.9% of F'8 *gal* DNA.

As a direct check of the correction factor under conditions where a large excess of heterologous DNA is present, ^{14}C -labelled F'8 *gal* DNA was purified by 3 cycles of equilibrium centrifugation in caesium chloride. Membranes carrying unlabelled strain 9-3 DNA or strain 9-3 DNA containing a small proportion (1.6%) of F'8 *gal* DNA were prepared, and annealed with ^{32}P -labelled cRNA as before. The difference between the amounts of RNA hybridizing with the two types of membrane represents the hybridization to F'8 *gal* DNA, at a level of 1.6% (Fig. 12). This may be compared directly with the hybridization to the F'8 *gal* DNA in DNA from strain 9-3 (F'8 *gal*) by subtracting the hybridization to strain 9-3 DNA from that to strain 9-3 (F'8 *gal*) DNA (Fig. 12). Throughout the curves there is a difference of about threefold in the amounts of hybridization. By measuring the difference between the extrapolated saturation values for strain 9-3 DNA with and without added strain F'8 *gal* DNA, a saturation value of 34% was calculated for the F'8 *gal* DNA. Correction of the earlier results on this basis gives an F'8 *gal* DNA content of 4.4–4.7% for the exponentially growing culture of strain 9-3 (F'8 *gal*).

In exponential growth with a generation time of 50 min *E. coli* contains an average of one replication fork/chromosome (Maaløe & Hanawalt, 1961; Cooper & Helmstetter, 1968). The DNA content/chromosome is therefore about $1.4 \times 2700 \times 10^6 = 3800 \times 10^6$ daltons (Sueoka & Yoshikawa, 1965). Since the genetic complexity and presumably the size of F'8 *gal* DNA is 76×10^6 daltons, if there

were one copy of the episome per chromosome, the episomal DNA would form 2% of the total DNA of the cell. The estimates of DNA content we have made (3.7–3.9, 4.4–4.7%) show that there are 1.9–2.3 copies of the episome/chromosome.

Galactose enzyme activities. Strains of *E. coli* containing a sex-factor carrying *lac*⁺ were found to have unexpectedly high concentrations of β -galactosidase (Revel & Luria, 1963; Jacob & Monod, 1961). This observation gave rise to the idea that an exponential culture contains two copies of F'*lac*/replicating chromosome. Similar findings were reported by Revel (1965) and by Falkow *et al.* (1964). Here we have shown directly that there are two copies of F'8 *gal* per cell. It is therefore of interest to compare the activities of the galactose enzymes in strains with and without F'8 *gal*.

Although we know the number of copies of F'8 *gal*, and therefore the number of episomal *gal* genes/chromosome, we do not know with certainty the average number of copies of the chromosomal *gal* gene. During exponential growth with a generation time of 50 min the average replicating *E. coli* chromosome contains about 1.44 genome equivalents of DNA. The expected frequency/cell of any gene depends (a) upon the mode of replication

assumed and (b) the position of the gene relative to the origin of replication (Sueoka & Yoshikawa, 1965). If different clones of the culture have different origins, and these are randomly determined, there will be about 1.4 copies of every gene per replicating chromosome, on average. If the origin of replication is fixed, the average number of copies of a gene falls as its map location is removed further and further from the origin. Recent experiments suggest that in strains of *E. coli* the direction of replication is clockwise and the origin is fixed, lying somewhere between *xyl* and *lys* (Wolf, Newman & Glaser, 1968; Caro & Berg, 1969). If so, the expected number of copies of *gal* per replicating chromosome is 1.35 (*xyl*) or 1.60 (*lys*). These three expectations do not differ greatly, and so we will assume for the moment the figure of 1.4.

Concentrations of galactokinase and the transferase enzyme in wild-type and homogenote strains are shown in Table 2. It is immediately evident that when the cells are induced with galactose the enzyme contents do not parallel the expected changes in gene frequency. When the cells are not induced (glycerol) or induced with the gratuitous inducer D-fucose, the agreement is better. A likely explanation is that in the presence of galactose, expression of the galactose operon is repressed in

Table 2. *Enzyme contents in wild-type (E1) and homogenote [E1(F'8 gal)] cultures induced with galactose or fucose, or not induced*

Values in columns 1, 2, 4 and 5 show enzyme activity in units/mg of soluble protein. Values in columns 3 and 6 show the ratio of the specific activities of the homogenote and wild-type extracts, and the ratio of the numbers of gene copies based on the assumption that there are 2 episomal genes/1.4 chromosomal genes.

Inducer	Kinase			Transferase		
	<i>gal</i> ⁺	<i>gal</i> ⁺ (F <i>gal</i> ⁺)	<i>gal</i> ⁺ (F <i>gal</i> ⁺)	<i>gal</i> ⁺	<i>gal</i> ⁺ (F <i>gal</i> ⁺)	<i>gal</i> ⁺ (F <i>gal</i> ⁺)
			<i>gal</i> ⁺			<i>gal</i> ⁺
Galactose	12.4	14.2	1.1	14.1	18.2	1.3
Fucose	12.5	19.9	1.6	12.4	37.7	3.0
None	0.8	3.1	3.9	1.2	4.0	3.3
Gene copies			2.4			2.4

Table 3. *Enzyme ratios in different strains*

Each value shows the ratio of the enzyme activity in the extract of a homogenote to that in the extract of the corresponding strain without an episome. The strains were: Epimerase⁻: NA9 (*try his str*^R *gal* E9) and 9S1 (*try his str*^R *gal* E9/F'8 *gal* E9); kinase⁻: NR3 (*try his str*^R *gal* K3) and 3S1 (*try his str*^R *gal* K3/F'8 *gal* K3), R⁻: TMG 6 (*try his str*^R R-6 *gal*⁺) and TMG 6/F'8 (*try his str*^R R-6 *gal*⁺/F'8 *gal*⁺). n.d.=not determined.

Inducer	Strain ...	Kinase			Transferase		
		Epimerase ⁻	Kinase ⁻	R ⁻	Epimerase ⁻	Kinase ⁻	R ⁻
Galactose		1.9	—	1.2	2.7	2.9	1.1
Fucose		2.5	—	n.d.	1.8	1.8	n.d.

Table 4. *Enzyme contents in homogenote and exogenote cultures*

Each value shows the average of the number of separate determinations shown in parentheses. In each case the value given represents the ratio of the enzyme activity of the strain in question to that of the wild type carried out at the same time. The strains used were: *gal*⁺ (F*gal*⁺), El (F'8) (*try his str*^R *gal*⁺/F'8*gal*⁺); Δ *gal* (F*gal*⁺), 9-3 (F'8) (*try his str*^R Δ *gal*/F'8*gal*⁺). The gene frequency ratios are calculated on the assumption that there are 2 episomal genes/1.4 chromosomal genes.

	<i>gal</i> ⁺ (F <i>gal</i> ⁺)			Δ <i>gal</i> (F <i>gal</i> ⁺)		
	Kinase	Transferase	Average	Kinase	Transferase	Average
Fucose	2.33±0.18 (3)	2.52±0.17 (4)	2.42	1.23±0.02 (4)	1.36±0.02 (4)	1.29
None	3.82±0.16 (6)	2.79±0.12 (6)	3.30	1.75±0.08 (6)	1.16±0.02 (6)	1.45
Gene frequency ratio			2.4			1.4

some way by a product of galactose metabolism. The repression effect would increase when the number of gene copies and the metabolism of galactose is increased. This interpretation is supported by experiments with strains carrying *gal*⁻ mutations on both the bacterial chromosome and the episome (Table 3). In these cases the ratio of the enzyme activity of the homogenote to that of the same strain without an episome was high, that is, similar to the ratio found (Table 2) when the wild-type was induced with fucose. Apparently the blockage of galactose metabolism eliminates the repression effect in these strains. Table 3 also shows an experiment in which constitutive (R⁻) strains were induced with galactose. Here the enzyme activity ratio between homogenote and non-homogenote was low, suggesting that the repression effect is not mediated by the *gal* repressor.

In a further series of experiments, the wild-type strain, the homogenote and a strain in which the entire galactose operon is deleted, but carrying *gal* on the episome, were all investigated after growth in glycerol, or in glycerol supplemented with D-fucose (Table 4). The observed proportionality in the enzyme contents was in reasonably good agreement with the expected frequencies of copies of the *gal* operon in the three strains.

DISCUSSION

The observed kinetic complexity of F'8 *gal* is in good agreement with molecular-weight determinations that have been carried out on other substituted sex-factors. It is similar in size to the F'*gal att*^A and F'*lac* studied by Freifelder (1968), larger than F (Freifelder, 1968; Falkow & Citarella, 1965) and the F'*gal* of Freifelder (1968) and F'13L (Falkow & Citarella, 1965), and smaller than FColVColB*trycys* (Hickson, Roth & Helinski, 1967). An objection might be raised against our measurement if, in *Proteus*, F'8 *gal* became dissociated into two separate replicons with different rates of multiplication. This in fact, is found to occur when

resistance transfer factors are transferred to *Proteus* (Rownd, 1969; Falkow *et al.* 1969). If so, the measured rate-constant of renaturation could not be related directly to the complexity of the DNA. There is, however, no evidence suggesting that F behaves in this way.

The saturation value extrapolated from the hybridization of strain F'8 cRNA with strain 9-3 DNA is close to 0.5%. This shows that about 1.3% of the *E. coli* DNA is homologous to F'8 DNA (multiplying by 2.6; see Bishop *et al.* 1969). Since most of the *E. coli* DNA carried by F'8 is deleted in strain 9-3, this suggests that close to 1.3% of *E. coli* DNA is homologous to F DNA, a value similar to the 1.9% suggested by Falkow & Citarella (1965). This type of experiment does not allow us to determine what proportion of F'8 DNA is homologous to *E. coli*.

The finding that there are two copies of the episome per replicating *E. coli* chromosome, or 1.4 copies per genome equivalent of *E. coli* DNA, during exponential growth with a doubling time of 50 min, is in full agreement with the suggestion (Jacob, Brenner & Cuzin, 1963) that replication of the episome and the chromosome is initiated simultaneously. Since the episome is so small, its replication will be completed within 3% of the time taken by the replication fork to traverse the chromosome. The sensitivity of the hybridization method used here is such that it lends itself to the further study of episome replication in exponential and synchronous cultures. Indeed, it provides a general method for measuring the number of copies of an episome present when episomal and host DNA have the same (G+C) content, provided only that the episome DNA can be transferred to *Proteus*.

The reason for the diminished contents of the galactose enzymes found in the presence of galactose is not clear. The effect is not observed when galactose metabolism is impossible, showing that it is not due to galactose itself, galactose 1-phosphate or UDP-galactose, and that it is probably distinct from the glucose effect (Paigen, Williams &

McGuiness, 1967). Our results further show that the repression effect is not mediated by the repressor of the galactose operon specified by the R gene. Clearly, one or more metabolic products of galactose metabolism are involved. The repression intensifies as the capacity of the cells to metabolize galactose is increased by increasing gene dosage. This suggests that we may be observing here 'true' catabolite repression.

The good correspondence found between the number of gene copies and the galactose enzyme contents during growth in glycerol medium is not unexpected. It means simply that if repression mechanisms are operating, they are operating to the same extent however many genes are present, and this is consistent with the fact that a metabolic product beyond the epimerase causes the repression observed in the presence of galactose. Therefore it would seem that the enzyme contents may be taken as a guide to the number of galactose genes present during growth in glycerol medium.

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REFERENCES

- Alberts, B. M. & Doty, P. (1968). *J. molec. Biol.* **32**, 379.
 Bishop, J. O. (1969). *Biochem. J.* **113**, 805.
 Bishop, J. O. (1970). *Biochem. J.* **116**, 223.
 Bishop, J. O., Robertson, F. W., Burns, J. A. & Melli, M. (1969). *Biochem. J.* **115**, 361.
 Britten, R. J. & Kohne, D. E. (1966). *Yb. Carnegie Instn Wash.* **65**, 73.
 Buttin, G. (1963). *J. molec. Biol.* **7**, 164.
 Caro, L. C. & Berg, C. M. (1969). *J. molec. Biol.* **45**, 325.
 Cooper, S. & Helmstetter, C. E. (1968). *J. molec. Biol.* **31**, 519.
 Falkow, S. & Citarella, R. V. (1965). *J. molec. Biol.* **12**, 138.
 Falkow, S., Haapala, D. & Silver, R. P. (1969). In *Bacterial Episomes and Plasmids*, p. 136. Ed. by Wolstenholme, G. & O'Connor, M. London: J. and A. Churchill Ltd.
 Falkow, S., Marmur, J., Carey, W. F., Spilman, W. M. & Baron, L. S. (1961). *Genetics*, **46**, 703.
 Falkow, S., Wohlheiter, J. A., Citarella, R. V. & Baron, L. S. (1964). *J. Bact.* **87**, 209.
 Fraser, S. & Jerrel, J. (1953). *J. biol. Chem.* **205**, 291.
 Freifelder, D. (1968). *J. molec. Biol.* **35**, 95.
 Geiduschek, E. P. (1962). *J. molec. Biol.* **4**, 467.
 Gillespie, D. & Spiegelman, S. (1965). *J. molec. Biol.* **12**, 829.
 Hickson, F. T., Roth, T. F. & Helinski, D. R. (1967). *Proc. natn. Acad. Sci. U.S.A.* **58**, 1731.
 Hirota, Y. & Sneath, P. H. A. (1961). *Jap. J. Genet.* **36**, 308.
 Jacob, F. & Adelberg, E. A. (1959). *C. r. hebdom. Séanc. Acad. Sci., Paris*, **249**, 189.
 Jacob, F., Brenner, S. & Cuzin, F. (1963). *Cold Spring Harb. Symp. quant. Biol.* **28**, 329.
 Jacob, F. & Monod, J. (1961). *J. molec. Biol.* **3**, 318.
 Kohne, D. E. (1968). *Biophys. J.* **8**, 1104.
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
 Maaløe, O. & Hanawalt, P. C. (1961). *J. molec. Biol.* **3**, 144.
 Marmur, J. & Doty, P. (1962). *J. molec. Biol.* **5**, 109.
 Miyazawa, Y. & Thomas, C. A., jun. (1965). *J. molec. Biol.* **11**, 223.
 Nakamoto, T., Fox, C. F. & Weiss, S. B. (1964). *J. biol. Chem.* **239**, 167.
 Paigen, K., Williams, B. & McGuiness, J. (1967). *J. Bact.* **94**, 493.
 Revel, H. R. (1965). *J. molec. Biol.* **11**, 23.
 Revel, H. R. & Luria, S. E. (1963). *Cold Spring Harb. Symp. quant. Biol.* **28**, 403.
 Rownd, R. (1969). *J. molec. Biol.* **44**, 387.
 Shapiro, J. A. & Adhya, S. L. (1969). *Genetics*, **62**, 249.
 Studier, F. W. (1965). *J. molec. Biol.* **11**, 373.
 Sueoka, N. & Yoshikawa, H. (1965). *Genetics*, **52**, 747.
 Thrower, A. J. & Peacocke, A. R. (1968). *Biochem. J.* **109**, 543.
 Wells, R. & Birnstiel, M. (1969). *Biochem. J.* **112**, 777.
 Wetmur, J. G. & Davidson, N. (1968). *J. molec. Biol.* **31**, 349.
 Wolf, B., Newman, A. & Glaser, D. A. (1968). *J. molec. Biol.* **32**, 611.