Fractionated Strands of Bacterial Deoxyribonucleic Acid

III. Transformation Efficiencies and Rates of Phenotypic Expression¹

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Preferential binding of guanine-rich ribopolymers to one of the complementary strands of denatured deoxyribonucleic acid (DNA) of *Diplococcus pneumoniae* permitted the fractionation of the complements in CsCl density gradients. Phenotypic expression of the newly acquired genes for four drug resistances was more rapid in cells transformed by the heavy fractions than in those transformed either by light fractions or by unfractionated DNA. The efficiencies of transformation with the two complements were nearly equal for the four markers tested. Both efficiency and expression results were the same whether we assayed the residual activity or the activity obtained by annealing the fractions with excess recipient DNA.

Guild and Robison (5) demonstrated fractionation of the complementary strands of pneumococcal transforming deoxyribonucleic acid (DNA) in alkaline CsCl density gradients. The complements differed with respect to the rate of phenotypic expression of the novobiocin resistance (*nov-r*) marker and with respect to message transcription (Miller and Guild, Abstr. Biophys. Soc., WG3, 1965; *in preparation*).

Both strands were active in transformation and were of comparable efficiency for transferring the *nov-r* marker. Although the streptomycin resistance (*str-r*) marker showed better renaturation when light and heavy fractions were mixed before annealing, no difference in expression time could be demonstrated (Green and Guild, *unpublished data*); therefore, it seemed possible that only one of the complements was active. Gabor and Hotchkiss (3) reported an apparent difference in efficiency of strands separated on methylated albumin-kieselguhr (MAK) for the *str-r* and erythromycin (*ery-r*) markers.

The better resolution of strands afforded by reacting them with guanine-rich ribopolymers (9) has enabled us to demonstrate expression-time differences for four drug-resistance markers and to show that for all four, including *str-r* and *ery-r*, the respective complements are of nearly equal efficiency.

¹ Parts of this study have been abstracted from a Ph.D. dissertation (Duke University, 1968) by J. M. P.

MATERIALS AND METHODS

DNA extractions. DNA extractions were performed essentially according to Marmur (7). Median single-strand molecular weights for DNA of strains 5MC and 8M are 4.1×10^6 and 10^6 , respectively, as determined by alkaline sedimentation velocity (10).

Bacterial strains. *Diplococcus pneumoniae* donor strains 5MC and 8M, bearing *str-r*, *nov-r*, *ery-r*, and bryamycin (*bry-r*) markers, and the drug-sensitive recipient strain Rx-1 are described elsewhere (Cato and Guild, J. Mol. Biol., *in press*).

Polyribonucleotide-mediated fractionations. Ribopolymers were purchased from Miles Chemical Co., Division of Miles Laboratories, Elkhart, Ind. $S_{20,w}^{50}$ determined in SSC (0.15 M NaCl, 0.015 M sodium citrate) for poly G was 11.0, and for poly (G, U) was 7.4 (G, guanosine; U, uridine).

Different types of DNA were mixed with ribopolymer preparations at polymer to DNA ratios of 0.25 to 1.0 (absorbance ratios at 258 nm), heated in screw-capped vials in a boiling-water bath for 10 min, and quenched in ice water. DNA concentration during heating was 160 to 190 $\mu\text{g}/\text{ml}$ in SSC. Cesium chloride (57 to 58%, w/w) was added, and the mixtures were centrifuged in polyallomer tubes with an overlay of mineral oil for 70 to 100 hr at 35,000 rev/min and 20 to 23°C in the SW-39 rotor.

Fractions (one drop each, 0.033 ml) were collected into 0.5 ml of 0.9 $\times$ SSC, 0.01 M tris(hydroxymethyl)-aminomethane chloride, pH 7.4. Absorbance of the resulting fractions at 258 nm was determined. Light and heavy fractions demonstrated their complementarity by regaining native characteristics only when mixed before annealing.

Transformation assays. Media, preparation of competent recipient cultures, and standard procedure for transformation have all been described elsewhere (4, 5).

DNA was limiting in assays for relative activity. For expression assays, higher concentrations were used (see figure legends), and the transformed cells were plated on drug-containing blood-agar at various times.

RESULTS

A typical set of expression curves for poly G-fractionated pneumococcal DNA is shown in Fig. 1. Light and heavy fractions were chosen from the respective peaks ($\rho = 1.716$ g/ml and $\rho = 1.754$ g/ml) of the well-resolved preparative run shown in Fig. 2 and were annealed with unfractionated recipient DNA to restore secondary structure. The unfractionated control sample was simply heat-denatured and annealed alone. The more rapid phenotypic expression of all markers introduced on the heavy strand is

apparent, both from the offset of expression curves (shown here only for *ery-r*, but similarly found for the other three drug markers) and from the progressive decrease in the ratio of heavy to light fractions with time. The ratio plot is the more valid representation, since it is not subject to the uncertainty of normalizing to a single assay point.

Large differences in efficiency of the complementary strands would result in an offset of the curve for unfractionated DNA toward that of the complement of greater efficiency. In the three cases in which we have good data (*nov-r*, *ery-r*, and *bry-r*), the expression of the unfractionated activity in fact matched fairly well the average expression of the heavy and light activities as found for *nov-r* by Guild and Robison (5). (The short time during which the drop in the ratio of heavy to light fractions for *str-r* occurs precludes obtaining sufficient resolution to determine whether the curve for unfractionated DNA

FIG. 1. Phenotypic expression. Heavy (no. 59) and light (no. 82) fractions from the poly G-mediated fractionation of Fig. 2 were each mixed with twice as much recipient DNA, heated for 10 min at 100 C, and then held at 65 C for 4 hr. The unfractionated sample (20 μ g/ml in SSC) was heated for 15 min at 100 C and then annealed for 7.75 hr at 65 C. All samples were then diluted 200-fold into the recipient cultures at time zero. Uptake of DNA was ended by the addition of deoxyribonuclease at 15 min. The abscissa represents the time of challenge by plating on agar containing the drug. The ordinate represents relative numbers of colonies observed for erythromycin resistance, normalized to the 90-min points. The insets compare directly the numbers of resistant colony-forming units, as the ratio of heavy to light fractions (H/L) versus time of challenge. Symbols: heavy ($\bullet$), unfractionated ($\square$), light ($\circ$).

matches the average of heavy and light fractions.) By this criterion, the efficiencies of the complementary strands appear to be about equal.

Poly (G,U)-fractionated DNA gave similar resolution of fast- and slow-expressing activities, with the heavy strand always producing more rapid expression.

Determination of the distribution of marker activities among the fractions is a more sensitive approach to the relative efficiencies of the respective complements. A complete marker assay is shown in Fig. 2. Samples of the fractions were annealed with excess recipient DNA to restore secondary structure, and transformation assays were done at concentrations on the linear portion of the dose curve. The data are analyzed in Table 1.

For the purpose of this analysis, the absorbance and marker profiles were divided at the point indicated by the arrow in Fig. 2 into light and heavy portions. The total absorbance is weighted somewhat to the heavy side. Correction for absorbance due to poly G, which represents

about 20% of the heavy fractions (Peterson and Guild, *in preparation*), indicates that the DNA is about equally divided. No attempt was made to remove poly G from the assay mixture, since it

TABLE 1. *Distribution of DNA and of renatured marker activities*

Determination	Fractions	
	Heavy (40-70)	Light (71-89)
Absorbance		
At 258 nm.....	54.8 ^a	45.2
At 258 nm (corrected for poly G).....	48.9	51.1
Transforming activity		
Streptomycin resistance.....	48.7	51.3
Novobiocin resistance.....	50.9	49.1
Erythromycin resistance.....	54.4	45.6
Bryamycin resistance.....	41.7	58.3

^a Per cent of total absorbance or activity summed over fractions indicated. See Fig. 2.

FIG. 2. *Fractionation of DNA and marker activities (renatured).* DNA from pneumococcal strain 5MC was denatured in the presence of one-fourth as much poly G. A portion of each fraction was mixed with two volumes of denatured recipient DNA (80 µg/ml in 2 × SSC), heated for 2 min at 100 C, and then held for 3 hr at 65. A total 500-fold dilution into the recipient culture was made at time zero. Deoxyribonuclease was added at 15 min. Time of challenge was 95 min. Symbols: streptomycin (□), novobiocin (■), erythromycin (○), bryamycin (●).

was found to have no significant effect on transformation, and since it is normally displaced from the DNA during the annealing procedure.

Summation of the marker activities in the light and heavy portions of the profiles shows that *str-r* and *nov-r* are divided nearly equally between the complements, whereas *ery-r* and *bry-r* show small biases of doubtful significance.

Similar results have been obtained with poly (G,U)-fractionated DNA, whether it was first annealed with recipient type DNA or merely reheated briefly to 100 C just before assay and not annealed with recipient DNA. In this latter case, detailed in Fig. 3 and Table 2, the assay was therefore of "residual" activities, the nature of which in *Pneumococcus* is somewhat in question and is still under study.

DISCUSSION

With the drug resistance markers assayed, the polymer-binding strands produced the more rapid phenotypic expression, showing that

TABLE 2. *Distribution of DNA and of residual marker activities*

Determination	Fractions	
	Heavy (25-51)	Light (52-74)
Absorbance		
At 258 nm.....	56.8 ^a	43.2
At 258 nm [corrected for poly (G, U)].....	50.3	49.7
Transforming activity ^b		
Streptomycin resistance.....	49.8	50.2
Novobiocin resistance.....	52.2	47.8
Erythromycin resistance.....	53.8	46.2
Bryamycin resistance.....	57.0	43.0

^a Per cent of total absorbance or activity summed over fractions indicated. See Fig. 3.

^b Since the interval between fractions assayed changes, one-sixth of the sum of fractions 57 through 72 was added to correct to a uniform three-fraction interval.

FIG. 3. *Fractionation of DNA and marker activities (residual)*. DNA from pneumococcal strain 8M was denatured in the presence of one-half as much poly G, U). After a 20-fold dilution in SSC, portions of fractions were heated for 5 min at 100 C, chilled, and diluted 10 fold into the recipient cultures at time zero. Deoxyribonuclease was added at 15 min. Time of challenge was 90 min. Symbols: streptomycin (□), novobiocin (■), erythromycin (○), bryamycin (●).

fractionation of fast- and slow-expressing activities is not a phenomenon peculiar to the *nov-r* marker, as might have been concluded from the studies of Guild and Robison (5) and Green and Guild (*unpublished data*) with DNA fractionated in alkaline CsCl. As suggested in these studies, failure to observe such fractionation of the other markers was probably due to lack of sufficient bias in the guanine plus thymine content of the complementary strands bearing these markers. The generality of the phenomenon and its correlation with transcription were also confirmed by the finding of Miller and Guild (Abstr. Biophys. Soc., WG3, 1965; *in preparation*) that pulse-labeled pneumococcal ribonucleic acid hybridizes preferentially with the light fractions of DNA from alkaline CsCl gradients.

Our data on both expression rates and distribution of marker activities among fractions, whether residual or renatured activity is assayed, indicate equal efficiency of strands. Since a particular transformation assay detects only a very small fraction of the total DNA, it is conceivable that an asymmetric physical distribution of the DNA bearing a marker could be compensated for by a difference in relative efficiencies as to spuriously give the observed distribution of marker activity. This would require, however, that much of both strands of a marker be bound by poly G, perhaps because of a switch in the direction of transcription nearby. If so, one would not expect a bimodal distribution, but a tendency for the marker to be of intermediate density. The low probability of such a close quantitative compensation, together with the fact that the four unlinked markers gave essentially the same result, rules out this explanation. Similar studies by Chilton (2) indicate that both complementary strands are equally efficient in transformation of *Bacillus subtilis*, whether assayed as single strands or after renaturation with recipient DNA.

Gabor and Hotchkiss (3), using chromatography on MAK to fractionate pneumococcal transforming DNA, reported that the more rapidly expressing component (strand fraction A) of the *str-r* marker is only half as efficient as the less rapidly expressing component, and that the other component (strand fraction B) is the more rapidly expressing for the *ery-r* marker and is only one-fifth as active as its complement. The *str-r* and *ery-r* markers were identical in the respective studies. The discrepancy between our results and those of Gabor and Hotchkiss may be related to the different methods of fractionation. The precise mechanism by which DNA is caused to fractionate on MAK is not known, but it is clear that the physical-chemical criteria in

the case of the *ery-r* marker are neither the same nor in the same relationship to transcription as those operative in polymer-mediated fractionation; nor indeed do they produce the same symmetry of fractionation of the marker. Strict correlation of fractionation on MAK with complementarity may not hold in the studies reported to date, for it has been shown that fractionation on MAK responds to various characteristics of the DNA which are not obviously related to complementarity, namely, guanine plus cytosine content, molecular weight, and secondary structure.

A possible source of the conflict may be the difference in the recipient strains used, since Rx-1 (our strain) and R-6 (theirs) differ in efficiency for *nov-r* (Guild, *unpublished data*) and for other markers (Lacks, *personal communication*), Rx-1 having high efficiency for all markers so far tested. Gurney and Fox (6) also reported that both strands of *ery-r2* are equally efficient; however, they used R-6 as recipient, which was presumably the same strain used by Gabor and Hotchkiss.

A useful conclusion can be drawn from the fact that both in this study and in alkaline CsCl fractionation where the fractionation is reversed, light versus heavy fractions, the expression differences give the same result with the residual activity and with the DNA annealed with unmarked recipient DNA. If the residual activity primarily results from cross-linked DNA strands of unequal length (1, 8), then the fractionation and expression properties are those of the larger of the two strands. Therefore, it is this strand which enters and participates in the transformation reaction, and this point may be of interest in elucidating the entry mechanism.

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