Adenine + Thymine Content of Different Genes Located on the Broad Host Range Plasmid RP4

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The genetic map of plasmid RP4 was correlated with its adenine+thymine (AT) map. For this purpose, RP4 DNA was digested with one or both of the restriction enzymes *Eco*RI and *Hin*dIII and the resulting linear RP4 molecules and fragments were partially denatured, examined in the electron microscope and their AT maps were determined using a computer program. From these AT maps the *Eco*RI and *Hin*dIII restriction sites were located on the AT map of RP4. Since the positions of these restriction sites on the genetic map of RP4 are known, the two maps could be compared. They revealed a high AT content for the Tn1 transposon and the kanamycin resistance gene. The *tra-1* region is also distinguished by a sharply defined AT-rich region, whereas *tra-2* and the tetracycline resistance gene have an AT content which is not distinctly different from the average AT content of RP4.

INTRODUCTION

RP4 is a resistance plasmid with remarkable features. It was isolated in 1969 from a *Pseudomonas aeruginosa* strain (Lowbury *et al.*, 1969) and it carries genes for ampicillin, kanamycin and tetracycline resistance and for its own replication, transfer (Datta *et al.*, 1971) and host fertility regulation (Pühler & Burkardt, 1978). It belongs to the incompatibility group IncP1 and is distinguished from other plasmids by its very broad host range (Datta & Hedges, 1972). Because of its wide interest, RP4 has been investigated in numerous molecular studies. The averaged guanine+cytosine (GC) content of the whole plasmid is 60% (Holloway & Richmond, 1973), which is high for R plasmid DNA. The ampicillin resistance gene is located on a transposon, called TnA or Tn1 (Hedges & Jacob, 1974). RP4 has been physically mapped by restriction enzyme cleavage analysis (DePicker *et al.*, 1977) and by partial denaturation (Burkardt *et al.*, 1978), and its genetic map has been constructed by transposon insertion mutagenesis (Barth & Grinter, 1977; Barth *et al.*, 1978). The aim of the present study was to correlate this genetic map with the adenine+thymine (AT) map of RP4 to determine the relative AT content of different genes located on this plasmid.

The technique of AT mapping, which we have previously used to investigate the relationship of RP4 to some other RP plasmids (Burkardt *et al.*, 1978), gives information on the distribution of AT- and GC-rich sequences along the DNA molecule. When the hydrogen bonds of double-stranded DNA are weakened (e.g. by treatment with alkali or heating) the double helix partially separates into its single strands. The separation occurs preferentially in AT-rich regions, and so the position of such sequences along the DNA molecule can be ascertained.

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METHODS

DNA isolation and treatment with restriction endonucleases EcoRI and HindIII. RP4 plasmid DNA was isolated from *Escherichia coli* NC22(RP4) as described previously (Burkardt *et al.*, 1978). The DNA was digested with the restriction enzymes as reported by Gautier *et al.* (1976).

Partial denaturation and preparation for electron microscopy. Partial denaturation was carried out according to Burkardt *et al.* (1978) with some modifications. To improve the quality of the single-strand preparation of partially denatured DNA, the formaldehyde, which was used for single-strand protection during denaturation (Inman, 1966), was subsequently removed. Cytochrome *c* was then added, and the sample was spread over formamide using the Kleinschmidt technique (Kleinschmidt & Zahn, 1959). The experimental details were as follows. (i) Removal of formaldehyde: a 20 μl sample of the mixture after partial denaturation was passed through a Sephadex G-50 column (50 mm × 5 mm diam.) which had been equilibrated with 1 mM-Na_2EDTA and was eluted with a similar solution. Three 20 μl samples of the DNA-containing fraction (located by previous calibration of the column with radioactively labelled DNA) were collected separately. (ii) Preparation of the spreading solution: 10 μl of a DNA-containing fraction from the column was mixed with 10 μl hyperphase resulting in a final solution of DNA in 0·1 M-Tris/HCl, 10 mM-Na_2EDTA and 30% (v/v) formamide (pH 8·5). [The formamide (Fluka, Buchs, Switzerland) was purified by recrystallization before use.] Just before spreading, 0·5 μl cytochrome *c* (5 mg ml^{-1}; Sigma, Type V) was added. (iii) Conditions of the hypophase: 10 mM-Tris, 1 mM-Na_2EDTA and 10% formamide (not purified) were adjusted to pH 8·7 with a few drops of 1 M-HCl. Parts of the hyperphase were picked up on parlodion-coated copper grids, positively stained with 10 μM-uranyl acetate in 90% (v/v) ethanol and rotary-shadowed with Pt/Ir at an angle of 6·5°. Lengths of native and denatured molecule segments were determined according to Burkardt *et al.* (1979). AT maps were constructed by the computerized evaluation method of Burkardt *et al.* (1978) with one modification: the resolving power of the program was increased to 0·25% of the total molecule length.

RESULTS

AT mapping of RP4 *DNA cleaved by EcoRI and HindIII restriction enzymes*

To correlate the AT map with the genetic map of the RP4 plasmid, it was necessary to know the positions of at least two markers on both maps. The restriction sites of the enzymes *Eco*RI and *Hin*dIII on the RP4 DNA were chosen for this, since each enzyme cuts the plasmid DNA only once and the cleavage sites are quite distant from each other and distributed asymmetrically. A typical *Eco*RI cleaved and partially denatured RP4 molecule is shown in Fig. 1. The distribution of the denaturation loops is not symmetrical and so an AT map with an unequivocal orientation of the peak pattern could be constructed (Fig. 2, upper part – as tandem repeat). This map shows a well-structured pattern with four fairly prominent main peaks. In a similar way, an AT map of *Hin*dIII-digested RP4 was constructed.

Alignment of the denaturation maps of molecules cleaved by EcoRI and HindIII

By partial denaturation of either *Eco*RI- or *Hin*dIII-cleaved RP4 plasmids, it was possible to locate the respective enzyme sites on the AT map. To determine the order of the peaks with respect to these cleavage sites, it was necessary to align one map to the other. The proper alignment could be found by shifting one map along the other (by computer) until the best fit was achieved. Figure 3 shows the 'fit' curves between the *Eco*RI map and *Hin*dIII map after shifting both maps along each other. The abscissa reflects the total molecule length and the ordinate indicates percentage congruence, i.e. the fraction of identical positions between both maps. The maximum congruence should show the point of best fit for both maps indicating the most probable shift, i.e. the distance between the *Eco*RI and *Hin*dIII sites. A value of about 75% congruence was found in the worst case, but four possible maximum positions were indicated by the computer: one at 0·35, one at 0·85, and two more, when the *Hin*dIII denaturation map was reversed, at 0·1 and 0·59. (The reason why the computer gives four possible positions is discussed later.) Since the distance between the *Eco*RI and *Hin*dIII sites is known to be 36% of the total molecule length

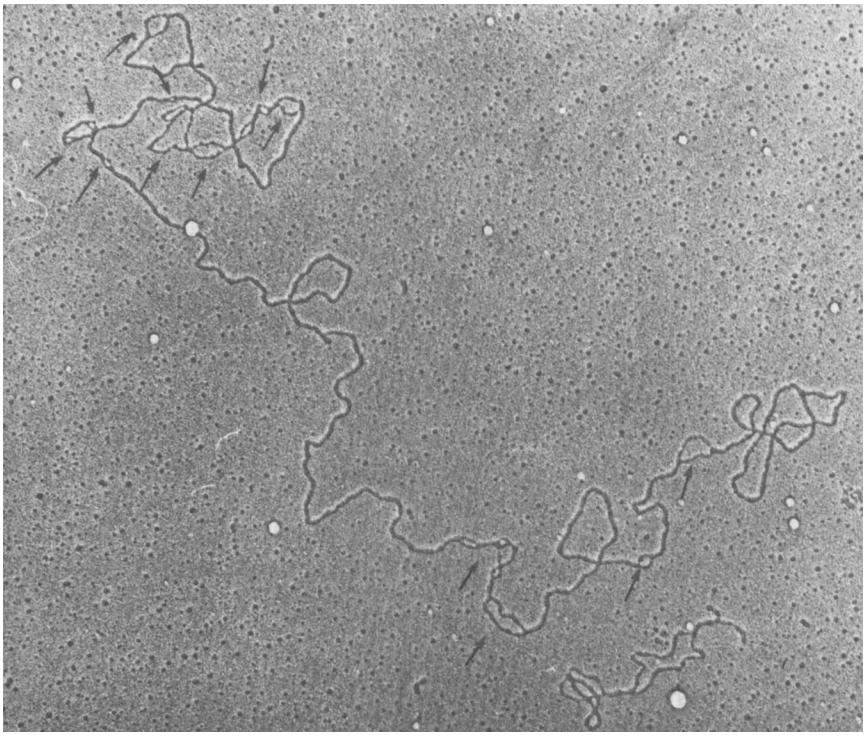

Fig. 1. Partially denatured RP4 DNA. The molecule was cleaved by the restriction enzyme *Eco*RI. Arrows point to denaturation loops. (Magnification ×23200).

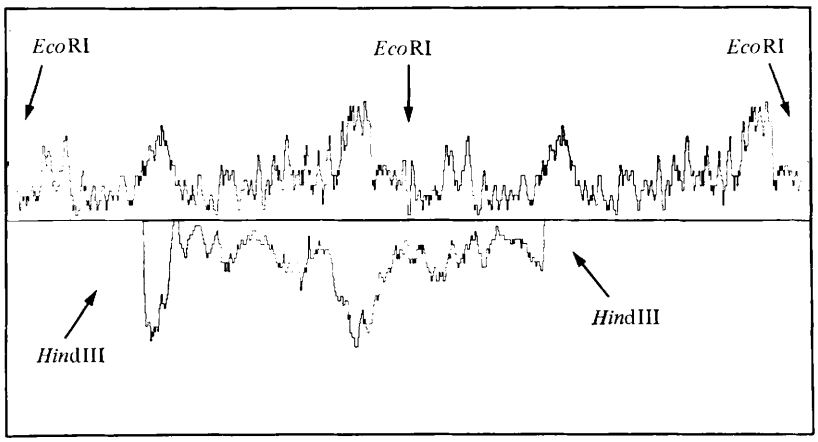

Fig. 2. Comparison of the partial denaturation patterns of RP4 DNAs cleaved by *Eco*RI (above) and *Hin*dIII (below). The RP4/*Eco*RI map is drawn as a tandem repeat.

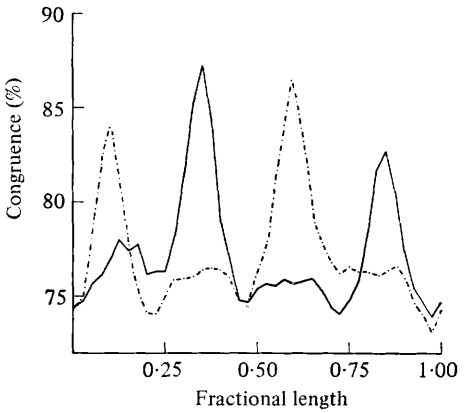

Fig. 3. Fit of two RP4 AT maps in relation to a computer shift to different positions (see text). The DNAs used for map construction were cleaved by *Eco*RI and *Hin*dIII. ——, Orientation of the denaturation peaks of both maps in one given direction; —·—·—·, orientation of the denaturation peaks of the *Eco*RI map in the same direction as before and of the *Hin*dIII map in the reverse direction.

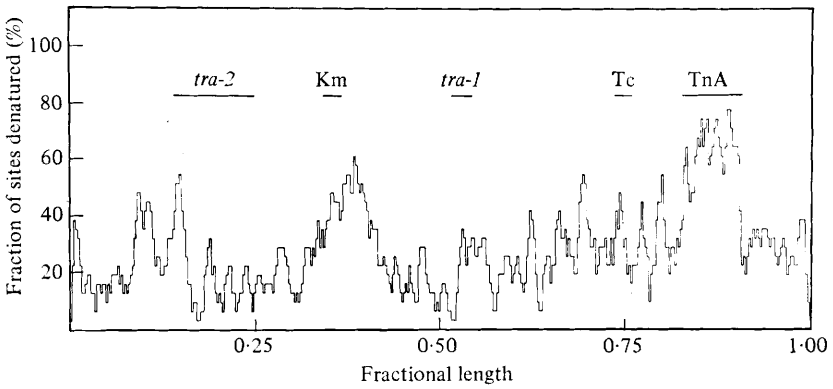

Fig. 4. Comparison of the partial denaturation pattern of RP4 DNA and the genetic map, both cleaved at the *Eco*RI site.

(DePicker *et al.*, 1977), two of the possible positions (0·1 and 0·85) can be discounted. The other two points suggested by the computer, corresponding to 35% and 41% of the molecule length between the *Eco*RI and *Hin*dIII sites, both fit within the range of error of the method. So the alignment of the partial denaturation peaks relative to these two sites ('left' or 'right') was still doubtful. Therefore, an experiment was carried out in which an RP4 molecule double digested by *Eco*RI and *Hin*dIII enzymes was partially denatured and the two *Eco*RI/*Hin*dIII fragments were aligned to the map of RP4 cleaved by *Eco*RI. The results unambiguously suggested the 0·35 shift to be correct, leading to the alignment of the *Eco*RI and *Hin*dIII maps shown in Fig. 2. The upper half of the diagram shows the RP4/*Eco*RI map in two adjacent copies; in the lower half the RP4/*Hin*dIII map (in one copy) has been shifted by 35%.

Correlation of AT map and genetic map by means of EcoRI and HindIII restriction sites

Having determined the positions of the restriction enzyme cleavage sites of *Eco*RI and *Hin*dIII on the AT map, and knowing the positions of these sites on the genetic map (DePicker *et al.*, 1977; Barth & Grinter, 1977; Barth *et al.*, 1978), the two maps could be correlated. In Fig. 4, the known genes of RP4 are shown in their appropriate positions

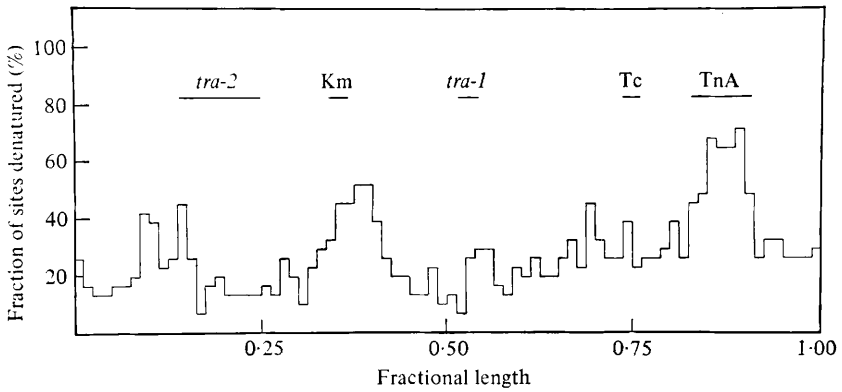

Fig. 5. Comparison of the partial denaturation pattern of an RP4 coarse map (see text) and the genetic map, both cleaved at the *Eco*RI site.

above the AT map. Both the genetic and physical maps start and end at the *Eco*RI cleavage site.

Some direct information about the AT content of the known RP4 genes can be obtained from Fig. 4. The part of the RP4 plasmid on which the transposon Tn1 is located coincides exactly with the most prominent AT peak (more than 80% of all molecules are denatured in this region). The kanamycin resistance gene is located in the next most prominent AT peak, whereas the AT content of the tetracycline resistance gene is moderate (20 to 50% of all molecules are denatured in this region) and is no different from the AT content of neighbouring regions of DNA. *Tra-1* (at 0·5 fractional length) has moderate AT content (30 to 40% of all molecules are denatured in this region) but is well-separated from the adjacent DNA regions, which have a low AT content (5 to 20% of all molecules are denatured). *Tra-2* (at 0·25 fractional length) appears to be composed of an AT-rich and an AT-poor segment.

Peak relevance of the AT maps

The AT map in Fig. 4 shows four or five well-defined major peaks and many small peaks. This raises the question of which of the AT peaks are really significant and which may only be background. To answer this question, a second evaluation method was applied. The numbers of denatured sites of five adjacent molecule segments (0·25% size) were averaged and a new map was constructed based on these calculations. The new map was five times coarser but eliminated some small background peaks (Fig. 5). Since the new map showed the same relevant peaks for the AT content of the marked genes (Fig. 4), the statements about the AT composition of RP4 genes still apply.

DISCUSSION

The method used for correlating the physical AT map and the genetic map depends very much on the reliability of the localization of the restriction enzyme sites of *Eco*RI and *Hin*dIII on the RP4 plasmid and so special attention was paid to this. The four possibilities for alignment of the *Eco*RI- and *Hin*dIII-cleaved molecules arose because the AT maps exhibit two major peaks, one of which is almost in the middle of the map and the other almost at the end. As can be seen in Fig. 2 (upper part), in which two adjacent copies are depicted, this symmetrical distribution of the main peaks leads to similar distances between them, thus resulting in two possible alignments for each of the two possible orientations as indicated by the 'fit' curves (Fig. 3).

The AT maps of the *Eco*RI- and *Hin*dIII-cleaved RP4 molecules exhibit some differences

in the shape of the peaks, whereas the peak positions are in good agreement. In general, the AT map of RP4 cleaved by *Hin*dIII seems to be less well-structured than the map of *Eco*RI-cleaved RP4. The problem of the peak shape is certainly due to our evaluation system and has been discussed previously (Burkardt *et al.*, 1978). The lower resolution of the *Hin*dIII map (Fig. 2) could be caused by a slightly higher degree of denaturation of the *Hin*dIII-cleaved DNA, leading to confluence of small adjacent denaturation loops to one larger loop. In thermal denaturation experiments, it is always very difficult to reproduce the denaturation conditions exactly.

Our mapping results revealed three RP4 plasmid regions which were well-defined by their AT pattern, namely, the Tn1 transposon, the kanamycin resistance gene and the transfer-1 region. The regions of the first two were also distinguished by a relatively high AT content. One can therefore speculate that the Tn1 transposon and the kanamycin resistance gene were not part of a hypothetical primitive RP4 plasmid but were acquired by this plasmid during its evolution. For Tn1, this could happen quite easily, since transposon structures can switch on to various replicons (Hedges & Jacob, 1974). Because of their high AT content, the Tn1 transposon and the kanamycin resistance gene might have originated not from *P. aeruginosa* (66% GC; Marmur & Doty, 1962), from which the RP4 plasmid was originally isolated, but rather from other bacteria with less dense DNA, e.g. Enterobacteriaceae (37 to 57% GC; Marmur & Doty, 1962). The two *tra* gene regions are separated from each other and exhibit different AT composition. Whereas *tra-1* is clearly different from its neighbouring regions, *tra-2* is composed of parts with different AT contents. This could indicate different origins of *tra-1* and *tra-2* and different origins of the *tra-2* components.

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