**SHORT COMMUNICATION**

**RNA Polymerase Binding Sites on the Broad Host Range Plasmid RP4**

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Binding sites of *Escherichia coli* RNA polymerase on RP4 plasmid DNA were determined electron microscopically. Comparison of the RNA polymerase binding map and the genetic map of RP4 revealed several strong binding sites outside the well-known RP4 genes. RNA polymerase binding sites for the three antibiotic resistance genes were also detected. Two binding sites were observed for the *tra-1* region, whereas the *tra-2* and *tra-3* regions showed no prominent affinity for RNA polymerase. The genomic regions for the replication origins, *oriV* (for vegetative replication) and *oriT* (for transfer replication, equivalent to *rlx*), both exhibited strong binding to RNA polymerase, as did genomic regions which code for trans-acting replication functions (*trfA* and *trfB*).

**INTRODUCTION**

RP4, an R plasmid that carries resistance genes against the antibiotics ampicillin, kanamycin and tetracycline, has been one of the most widely studied large extrachromosomal elements because of its broad host range. We have characterized RP4 and similar plasmids by their partial denaturation pattern (Burkardt et al., 1978), investigated the relationship between different P-type plasmids by heteroduplex techniques (Burkardt et al., 1979) and determined the relative AT content of different RP4 genes (Burkardt et al., 1980). In this paper we report an investigation of the location of *Escherichia coli* RNA polymerase (RNAP) binding sites on RP4: we incubated RP4 DNA with RNAP, cleaved it with restriction enzymes to obtain reference markers and mapped the binding sites by electron microscopical analysis of the structural complex.

**METHODS**

**DNA isolation.** RP4 DNA was isolated as described previously (Burkardt et al., 1978). For the present experiments all DNA samples used had at least 50% supercoiled RP4 DNA, because RNAP binds preferentially to covalently closed circular DNA (data not shown). The ratio of open circular to covalently closed circular DNA was determined electron microscopically.

**Binding of *E. coli* RNAP to RP4 DNA.** In a test tube were mixed 0.09 ml double-distilled water, 0.01 ml incubation buffer, 1 µl RP4 DNA (70 µg ml⁻¹ in 10 mM-EDTA, pH 7) and 1 µl RNAP (30 µg ml⁻¹ in incubation buffer 1:10). The molar ratio of RNAP to DNA was therefore about 34:1. The incubation buffer consisted of 0.3 M-triethanolamine, pH 7.9, 0.5 M-KCl and 0.08 M-magnesium acetate. In a second series of experiments the concentration of RNAP was changed to 300 µg ml⁻¹ resulting in a 10-fold higher RNAP to DNA molar ratio. The mixture was incubated at 37 °C for 10 min. The RNAP was a gift from Dr W. Lotz, University of Erlangen.

**Cleavage with restriction endonucleases.** An EcoRI or HindIII preparation (2 µl) was added to the sample. The EcoRI preparation (a gift from Dr A. Rösch, University of Erlangen) had a concentration of about 0.5 enzyme units µl⁻¹. The HindIII preparation (Boehringer) was used as indicated by the manufacturer. The mixture was incubated at 37 °C for 30 min.
Fixation of the DNA/RNAP complex for electron microscopy. A fixation mixture of 10 μl double-distilled water, 5 μl incubation buffer (1:5) and 5 μl glutaraldehyde (2%) was prepared immediately before use. This mixture was added to the sample and the complex of cleaved RP4 DNA and RNAP was fixed for 10 min at 37 °C.

Sample preparation for electron microscopy. The sample was deposited on the surface of a clean plastic Petri dish to form a droplet. The droplet was touched with a piece of freshly cleaved mica. The mica was then washed twice by touching droplets of double-distilled water and the sample was stained by floating for 90 s on 1% (w/v) aqueous uranyl acetate. After two further washing steps, the sample was dehydrated stepwise by immersion in 50 and 90% (v/v) ethanol and then air-dried. The mica was rotary shadowed with platinum/iridium (80/20) at an angle of 5.5° and covered with a carbon film by carbon casting. To achieve better floating of this layer, the carbon rods were treated with a 0.5 M-NaCl solution before use; the carbon film was floated on to a water surface and parts were picked up with copper grids. The replicas were observed in the electron microscope.

RESULTS AND DISCUSSION

RNAP/RP4 binding assay

Using the direct mica adsorption method the RNAP molecules attached to RP4 DNA could be observed in the electron microscope. The RNAP molecules were distributed asymmetrically and non-uniformly along the RP4 DNA. The number of RNAP molecules bound depended on the ratio of RNAP to RP4 DNA. In the experiments with 10-fold increased RNAP concentrations 6–19 (average 12) RNAP molecules were found bound to RP4 DNA, whereas in the experiments with low RNAP concentrations the number was 3–8 (average 6).

Similar binding studies have previously been reported by several groups. Most work in this field has been done with T7 DNA (for review, see Koller et al., 1978); coliphages T2 (Cherny et al., 1977), #X174 and S13 (Rassart et al., 1979) have also been investigated.

To our knowledge, no similar studies with plasmid DNA have previously been done. Two difficulties with plasmid DNA might be responsible for this. (i) In contrast to studies with linear phage DNA, RNAP binds in vivo to supercoiled double-stranded DNA, but the DNA must be linear for evaluation. The affinity of RNAP for linear plasmid DNA is, however, much lower than for supercoiled circular DNA. We attempted to circumvent this problem by binding RNAP before cleavage of the DNA. Of course, we cannot be sure that all DNA/RNAP complexes were stable enough to survive the cleavage reaction. It is possible that some RNAP dissociated from the DNA during this process resulting in a reduction of the number of bound RNAP molecules. Nevertheless, as a statistical evaluation was applied, this reduction should also reflect the specific strength of an RNAP binding site. Both methods – binding and subsequent cleavage and vice versa – were compared elsewhere and somewhat more detailed maps were obtained with the technique described in this communication (R. Lurz, personal communication). (ii) Phages are known to carry very strong promoters, since it is essential for them that the host RNAP binds preferentially to the phage DNA. This need not be the case for plasmid DNA. These two points may account for the relatively high molar ratios of RNAP to DNA required in the present study (34:1 and 340:1), and these ratios should not be directly compared with lower ratios in phage experiments.

Mapping of RNAP binding sites

We tried to map RNAP binding sites on RP4 in relation to known restriction enzyme cleavage sites. For this purpose we chose EcoRI and HindIII, because both enzymes are known to cut RP4 DNA only once (Jacob & Grinter, 1975; DePicker et al., 1977). Figure 1 shows the maps obtained from experiments in which RP4 DNA was cut with HindIII (upper diagram) or EcoRI (lower diagram). The alignment of the two maps was achieved by computer, which proposed only one position of maximal map congruence in one of the two possible orientations, displaced by 0-35 molecule unit length. This corresponds perfectly with the distance between the EcoRI and HindIII sites known from the restriction maps. For
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Fig. 1. Comparison of the genetic map and RNAP binding maps of plasmid RP4. The zero point corresponds to the single EcoRI site on RP4. The ordinate scale indicates the percentage of RP4 molecules with an RNAP molecule at a given site. The lower binding map was derived from an experiment in which the RP4 DNA was cut by EcoRI (19 RP4 molecules); the upper binding map was from a similar experiment with HindIII (29 RP4 molecules). The HindIII map was aligned to the EcoRI map by computer, cut at its EcoRI site and rejoined at its HindIII ends (indicated by the arrow). The molar ratio of RNAP to RP4 DNA was 34:1 in the EcoRI experiment and 340:1 in the HindIII experiment.

The construction of the final diagram the original HindIII map was cut at its EcoRI site and rejoined at the HindIII site. Thus both maps start and end at the EcoRI site which is conventionally defined as the zero point in all physical RP4 maps. The ordinate scale in the diagrams indicates the percentage of RP4 molecules that carry one RNAP molecule at a given position. Therefore peaks on the map indicate RP4 DNA regions with high RNAP binding activity, whereas the deepest valleys on the map show sites where bound RNAP molecules were never observed. For map construction the diameters of the RNAP molecules were mathematically enlarged to 2% of the length of the RP4 molecule to make the peaks more prominent. We used a similar enlargement procedure for map construction by partial denaturation (Burkhardt et al., 1980). This procedure results in a map which is relatively coarse but it eliminates some faults of map point displacement caused by artifacts in preparing or measuring the complexes. As controls, we drew maps with 1% and 0.5% enlargement. The latter was identical with the resolution limit of the computer program. During the enlargement process virtually no peaks disappeared or emerged but some neighbouring peaks fused, resulting in a clear map with prominent RNAP binding positions.
Correlation of the RNAP binding map and the genetic map

Knowing the positions of the restriction sites of EcoRI and HindIII on the RNAP binding map, we were able to make a direct comparison with the genetic map obtained by transposon mutagenesis (Barth & Grinter, 1977; Barth et al., 1978; Barth 1979), on which these restriction sites were already known. In Fig. 1 we have marked the known RP4 genes on the HindIII RNAP binding map because this map was constructed by use of a high RNAP concentration, resulting in a more detailed RNAP binding pattern. We have also marked the genes known on RK2 (Thomas et al., 1979) because this plasmid is identical with RP4 (Burkardt et al., 1979).

Resistance genes. The strongest RNAP binding sites of the RP4 resistance genes lie in the Tn1 transposon and the tetracycline resistance gene region. The kanamycin gene apparently has the weakest RNAP binding site of the three resistance genes; a binding site peak only emerged in the experiments with high RNAP concentrations. The good binding properties of the Tn1 DNA and the tetracycline resistance gene were not unexpected, because both ampicillin and tetracycline resistance are well expressed genes not only in Pseudomonas aeruginosa (Lowbury et al., 1969), the original RP4-carrying host, but also in E. coli (Datta et al., 1971) and in soil bacteria (Pühler & Burkardt, 1978). It is questionable whether the low affinity of the kanamycin resistance gene has any meaning in vivo, because this resistance is equally well expressed. Within the Tn1 transposon a second RNAP binding site could indicate a second promoter. This is not very surprising because Tn3, a closely related transposon, codes for at least two different gene products (Heffron et al., 1978), and previous RNAP binding studies on Tn3 DNA are in full accordance with our findings (Vollenweider et al., 1979).

Transfer genes. The tra-2 and tra-3 segments fall in DNA regions with notably little affinity for RNAP, whereas the tra-1 segment is distinguished by two moderately active RNAP binding sites. The general low RNAP binding might correspond with the relatively low transfer frequencies of RP4 (Pühler & Burkardt, 1978) compared to derepressed plasmids like F.

Sites and genes for replication. Both ori sites (oriV and rlx) are situated in DNA regions with relatively high affinity for RNAP, especially oriV. Whether the small peaks exactly above these genetic regions do really reflect strong binding sites cannot be ascertained because of the roughness of the binding map. The trfA and trfB regions, which code for trans-acting replication functions, also lie in DNA segments that preferentially bind RNAP. The high affinity of RNAP for the ori regions could correspond to the RNA priming of DNA synthesis.

RNAP binding peaks in unknown genes. Near the EcoRI restriction site, especially left and right of the Tn1 transposon, very strong RNAP binding sites were detected which do not correlate with known RP4 genes. If they are identical with active promoters, then some new, as yet unknown and very active RP4 genes are indicated. Further experiments are required to clarify whether these binding sites actually play a role in mRNA synthesis.

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REFERENCES


