Discriminative Effect of Rifampin on RNA Replication of Various RNA Bacteriophages

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Rifampin interferes exclusively with RNA replication in vivo of the group I phages MS2, f2, and R17, whereas Qβ RNA replication is unaffected by the drug. In addition, rifampin has a discriminative effect of group I phage RNA replication. In the experimental system employed by us the antibiotic differentially interferes with the synthesis of minus RNA strands in f2, whereas it has almost no effect on the synthesis of progeny plus strands. In MS2, the drug differentially arrests the synthesis of progeny plus strands and almost fails to affect the synthesis of minus RNA strands. In R17 both steps of its RNA replication are affected by rifampin, although each step is only partially (approximately 50%) inhibited. The relation of the present results to the possible role of bacterial proteins and tertiary structure of phage RNA in the process of template recognition is discussed.

The antibiotic rifampin inhibits the initiation of RNA synthesis (21, 42), and subsequently protein synthesis (34), in Escherichia coli by acting directly on the beta subunit of the enzyme RNA polymerase (32). It was reported that this drug interferes with the intracellular growth of RNA phages (6, 7, 14, 24, 25, 31, 36) and, in addition, inhibits the release of mature phage particles from the host (7, 8). RNA phage synthesis and release are not affected by the antibiotic when the host possesses a rifampin-resistant RNA polymerase (8, 24, 31). This implies that host-specified components participate in phage development, and studies on the effect of the drug in each developmental stage may provide a useful tool for the understanding of a host-parasite relationship in an RNA phage-E. coli system.

Recently, we studied the effect of rifampin on the intracellular development of the RNA phage MS2 (6) and have shown that the drug interferes with MS2 RNA replication by the inhibition of the synthesis of progeny RNA strands. This inhibition is not immediate, but rather a period of about 15 min is required for the drug to exert its maximal inhibitory effect on MS2 RNA replication. As also reported (25, 36), RNA replication of M12 or R17 phages proceeds in a normal way only about 25 min after the addition of rifampin; later on the synthesis of both phage-specific RNA and proteins is simultaneously arrested. On the other hand, according to Passent and Kaesberg (31), Qβ phage RNA and phage protein synthesis is not interfered with by the drug, but the rate of assembly into phage particles was found to be low.

In this report we show that rifampin interferes exclusively with the RNA replication in vivo of the group I phages MS2, f2, and R17, whereas the replication of Qβ RNA is unaffected by the drug. Moreover, rifampin has a discriminative effect on the RNA replication of group I phages. Our data indicate that the antibiotic mainly interferes with the synthesis of minus RNA strands in f2 RNA replication; in MS2 it mainly arrests the synthesis of plus RNA strands; and in R17, both steps of its RNA replication are partially inhibited.

MATERIALS AND METHODS

Bacterial strains and phages. E. coli AB 301 (A) meth’ ribonuclease I-less was obtained from R. Gesteland. Phages were from the following sources: MS2 from R. Sinshheimer; f2 from N. Zinder; R17 from J. Argetsinger-Steitz; and Qβ from T. August. MS2, f2, and R17, but not Qβ, reacted serologically with anti-MS2. Qβ, but not f2, MS2, and R17, was able to grow on E. coli strain M27 obtained from J. Argetsinger-Steitz.

Buffers. Phosphate-buffered saline, pH 7.0, was made up as three separate solutions which were mixed cooled as described by Rappaport (33). STE buffer for chromatography on cellulose had the following composition: 0.1 M NaCl, 0.001 M EDTA, and 0.05 M Tris (pH 6.85).

Preparation of 3H-labeled phage. MS2, f2, R17, and Qβ with 3H-labeled RNA were prepared as
described by Erikson et al. (9) and purified according to Doi and Spiegelman (5).

**Infection, isolation of RNA, and determination of the amount of phage parental RNA in double-stranded form.** E. coli cells were grown in a tryptone medium with a shaking rate of 120 oscillations/min, at 37°C to a density of 2 x 10⁸ cells/ml, and infected with ³H-labeled RNA phage at a multiplicity of 5. At various times after infection the cells were rapidly cooled and washed four times with phosphate-buffered saline to remove the uninjected phages. The cells were then washed with 0.01 M Tris buffer, pH 7.75, containing 0.0015 M magnesium acetate and 30% sucrose, and resuspended in 0.5 ml of the same buffer. Lysozyme, 1 mg/ml, was added and the cells were twice frozen in a carbon dioxide-acetone bath and thawed in an ice bath. The spheroplasts obtained were lysed by the addition of 0.3 ml of 15% sodium dodecyl sulfate and were incubated for 3 min at 4°C. RNA was extracted as reported previously (1). The appearance of parental RNA in the double-stranded structure was determined by the method of Franklin (12), which is based on the finding that single-stranded RNA adsorbed to cellulose columns can be eluted with 15% ethanol-STE buffer, whereas RNA in double-stranded form is eluted from cellulose columns with STE buffer alone.

**RESULTS**

**Effect of rifampin on MS2 RNA replication.** The effect of rifampin on the appearance of parental RNA in double-stranded form after infection with ³H-labeled MS2 was studied. As shown (Fig. 1) in a bacterial culture not treated with the drug, the amount of parental RNA converted into the duplex increased until about 15 min postinfection and comprised 16 to 32% of the injected parental RNA. Approximately 15 min after infection the parental RNA became progressively displaced from the duplex, and by 40 min only 5% of it remained in this structure. When rifampin (100 μg/ml) was applied to the host culture 30 min before infection, phage synthesis was reduced by a factor of 20 (Table 1), but, as shown in Fig. 1a, the conversion of injected parental RNA into the duplex was almost unaffected and comprised about 80% of the maximum amount converted by the control. However, the displacement of the parental RNA from the duplex was completely arrested by the drug. A complete inhibition of parental RNA displacement was already achieved when rifampin was added at the time of infection (Fig. 1c), which was about 15 min before the beginning of this process.

The maximum amount of parental RNA converted into the double-stranded structure varied and mainly depended upon the batch of labeled phage used. Therefore, experiments on the effect of rifampin on the appearance of parental RNA in the double-stranded form were always carried out together with a control, untreated culture infected with the same batch of labeled RNA phage. The results thus obtained were invariably the same as in the case of MS2, where we found in four separate experiments a complete and preferential inhibition of parental RNA displacement from the duplex when the drug was added 30 or 15 min before or
at the time of infection (Fig. 1).

As is now generally accepted (30, 47), phage RNA replication proceeds in two steps: (i) synthesis of minus strands on parental plus template and (ii) synthesis of progeny RNA strands complementary to the minus strands. After infected cells are deproteinized for the isolation of RNA, the first step is manifested by the conversion of parental RNA into double-stranded form, and the second step by the displacement of parental RNA from the duplex (9, 46). We have already shown (6) that the parental MS2 RNA which accumulated as a double-stranded structure in the presence of rifampin sedimented as replicative form and replicative intermediate at about 12S and 16S to 23S, respectively. After denaturation of these double-stranded structures with formamide, the labeled parental RNA quantitatively co-sedimented with MS2 RNA. Thus, our experiments show that in the case of MS2 rifampin almost does not affect the synthesis of minus RNA strands but does seem to arrest the synthesis of progeny strands, and, therefore, the parental RNA remains quantitatively undisplaced from the double-stranded form.

**Effect of rifampin on f2 RNA replication.**

As demonstrated in Table 1, rifampin at the concentration of 100 \( \mu g/ml \) inhibited by the same values the synthesis of both MS2 and f2 phages. When the drug was applied at 30 min before or at the time of infection with either MS2 or f2, only about 5 and 10%, respectively, of the viable particles were formed. On the other hand, the effect of rifampin on f2 RNA replication was found to differ from that on MS2 RNA replication. As shown (Fig. 2a and b), application of the drug 30 or 15 min before infection with \(^{3}H\)-labeled f2 severely inhibited the conversion of the injected parental RNA into the double-stranded form. This process was inhibited up to 75%. The action of rifampin on the second step of f2 RNA replication was studied by its application to the bacterial culture at 10 min before or at the time of infection. As shown (Fig. 1a and b), when the drug was applied at these times the second step of MS2 RNA replication was completely inhibited. On the other hand, in the case of f2 (Fig. 2c and d), the displacement of its parental RNA from the duplex was almost unaffected by the antibiotic, and only a 20% inhibition of this process was observed. Thus, contrary to the effect of rifampin on MS2 RNA replication, the drug mainly inhibited the conversion of f2 RNA into the double-stranded structure, whereas the displacement of f2 RNA from the duplex was almost unaffected and, therefore, the antibiotic seemed to inhibit the synthesis of minus f2 strands and almost failed to affect the synthesis of f2 progeny RNA.

**Effect of rifampin on R17 RNA replication.**

Then we studied the effect of rifampin on the two steps of R17 RNA replication. The action of the drug on the synthesis of R17 minus RNA strands detected by studies on its effect on the conversion of R17 parental RNA into the double-stranded structure is illustrated in Fig. 3. This process was inhibited by the maximal

<table>
<thead>
<tr>
<th>Phage</th>
<th>Time of rifampin addition (min) relative to the time of infection</th>
<th>% Control yield</th>
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<tbody>
<tr>
<td>MS2</td>
<td>-30</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>f2</td>
<td>-30</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>R17</td>
<td>-30</td>
<td>4.2</td>
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<tr>
<td></td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Q8</td>
<td>-30</td>
<td>3.5</td>
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<td>0</td>
<td>8</td>
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*E. coli* cells were infected with the phages at multiplicity of 5 and treated with rifampin (100 \( \mu g/ml \)) either 30 min before or at the time of infection. Total phage yield was determined 40 min postinfection as reported previously (8).

**FIG. 2.** Effect of rifampin on the amount of f2 parental RNA in the double-stranded form at different times after infection. *E. coli* cells were infected with \(^{3}H\)-labeled f2 at a multiplicity of 5. Rifampin (100 \( \mu g/ml \)) was added (a) 30 min before, (b) 15 min before, (c) 10 min before, and (d) at the time of infection. All other procedures were as described in the legend of Fig. 1 and in Materials and Methods. Symbols: ◆, treated with rifampin; ○, control.
value of 50%, which was achieved when the drug was applied to the bacterial culture 30 min prior to infection (Fig. 3a). In addition, the drug also partially inhibited the second step of R17 RNA replication. A 50% inhibition of the displacement of parental R17 RNA from the duplex was observed (Fig. 3c and d). In five separate experiments a maximal inhibition of approximately 50% of each replication step was found. The inhibitory effect of the drug was not augmented by the increase of its concentration up to 200 μg/ml.

**Effect of rifampin on Qβ RNA replication.**
Qβ phage synthesis was blocked by the addition of rifampin to the host culture, either before or at the time of infection (Table 1), and the inhibitory values obtained were similar to those described for the RNA phages MS2, f2, and R17. However, in contrast to the inhibitory effect of the drug on RNA replication of those phages shown in the previous sections, Qβ RNA replication was found in our experiments to be unaffected by the antibiotic. We were unable to detect any inhibitory effect of rifampin, either on the conversion of the injected parental Qβ RNA into the double-stranded structure or on its displacement from the duplex (Fig. 4). No inhibition of Qβ RNA replication was observed, even when the antibiotic was applied to the bacterial culture as early as 30 min before infection (Fig. 4a).

**DISCUSSION**

The RNA-containing phages are classified into three to five serological groups (27, 37, 44). Group I comprises phages such as MS2, f2, and R17, which are quite similar to one another, primarily in their particle properties (39), and differ considerably from Qβ, a member of group III (29). These four pages have been studied intensively, but most of the information available on RNA replication comes from studies on Qβ (41). The present data clearly show that rifampin inhibits the in vivo RNA replication of phages belonging to group I (MS2, f2, and R17) whereas that of Qβ is unaffected by the drug. As demonstrated (8, 24), this inhibition is depend-
ent on the sensitivity of host RNA polymerase to the action of the antibiotic. It seems, therefore, that the inhibitory effect of rifampin on phage RNA replication is concerned with a host-specified component(s) exclusively involved in group I phage RNA replication versus that of Qβ. This component(s), presumably a protein(s), could be a host subunit of the RNA replicate itself, a factor needed for enzyme activity, or part of the replication site of group I phages.

Several bacterial proteins have been shown to participate directly in RNA replication. The core of the enzyme Qβ replicase contains, in addition to the phage-specified protein (subunit II), three bacterial proteins (subunits I, III, and IV) (18, 20). Subunits III and IV are the protein synthesis elongation factors Tu and Ts (2). Subunit I has been identified as a translational interference factor I (15) and more recently as 30S ribosomal protein S1 (43). In addition, two other bacterial proteins, factor F1 and F11, are required for the purified core of Qβ replicase to copy the parental plus strand (13, 38). Relatively little is known about the replicases of group I phages since they are unstable and difficult to purify away from the endogenous template RNA. However, recently the f2 poly G polymerase was extensively purified (10). Structurally, the f2 poly G polymerase appears to be similar to the core replicase of phage Qβ. Functionally, however, the Qβ and f2 enzymes differ significantly. The Qβ core replicase is able to use Qβ complementary strand, as well as poly C as template, and requires bacterial factors only for the activity with Qβ plus strands as RNA template. On the other hand, the highly purified f2 enzyme is active only in the presence of poly C template but is inactive with either f2 plus or f2 minus strands (10). Federoff and Zinder reported (11) on a f2 RNA-binding factor(s) from f2-infected cells which is required for f2 replicase activity with either f2 or f2 complementary strands as templates. The chemical identity of the f2-binding factor(s) has not yet been established, but the persistence relationship between f2 replicase activity and total bacterial protein concentration implicates bacterial protein(s). Of particular interest is their finding that host factor F1 and F11, required for core Qβ replicase, did not replace the f2 factor(s). These data taken together with ours on the exclusive inhibitory effect of rifampin on group I phage RNA replication versus that of Qβ indicate that a bacterial protein(s) involved in group I phage RNA replication is not required in the case of Qβ. This assumption is further supported by our recent finding of a temperature-sensitive host mutant which is defective for RNA replication of MS2, f2, and R17 and permits that of Qβ at the elevated temperature (R. Schoulaker and H. Engelberg, manuscript in preparation).

The inhibitory effect of rifampin on phage RNA replication can be explained by the requirement of a concomitant synthesis of a host protein(s) involved in this process of group I phages. This explanation assumes that the bacterial protein(s) concerned is a short-lived one as a result of either: (i) its structural or functional lability, which is presumably responsible for the extreme instability of RNA replicases induced by group I phages; or (ii) its existence in the bacterial cell as a limited pool of precursor molecules required for the formation of a stable cellular structure, such as ribosomes or membranes. An alternate explanation for the action of rifampin on RNA replication assumes that the group I phage replicating system and the host enzyme RNA polymerase compete for a bacterial protein. As reported (4), rifampin allows the formation of a core RNA polymerase-DNA complex, but prevents initiation of RNA polymerization. One is tempted to speculate that, in the presence of the drug, the complex E. coli RNA polymerase-transcriptional factor(s) remains bound to the DNA and is not released as in the normal transcriptional process. Consequently, a hypothetical transcriptional factor(s), which we assume to be limited in number, is unavailable to form an active RNA replicating complex.

Our results show as well that, even though rifampin does not interfere with Qβ RNA replication, it inhibits the synthesis of Qβ phage. These results are in agreement with the data of Passent and Kaesberg (31) and further support their assumption that a host protein(s) is probably required for Qβ phage assembly. Phage assembly can be interfered with by rifampin via one of the mechanisms suggested above for its action on RNA replication. Without excluding other possibilities, E. coli ribosomal or membranous proteins might be candidates for the phage assembly protein(s). Thus far we do not have evidence for a possible role of a bacterial protein(s) in the assembly of group I RNA phages.

Another principal finding of the present report is that even though rifampin inhibits the RNA replication of group I phages, it has a discriminative effect on the replication of f2, MS2, and R17 RNAs. With the method employed it was shown that the antibiotic differentially interferes in f2 with the synthesis of minus strands, whereas it has almost no effect on the synthesis of progeny plus strands. On the other
hand, in the case of MS2, rifampin differentially arrests the synthesis of progeny plus strands and almost fails to affect the synthesis of minus strands. In R17, both steps of its RNA replication were affected by the drug, although each step was only partially (approximately 50%) inhibited. These observations suggest that the action of rifampin on group I phage RNA replication is concerned with a bacterial protein(s) essential for the first step of f2 RNA replication and for the second of MS2. This bacterial protein(s) is only partially needed and refers to both steps of the RNA replication of R17. Our data do not rule out the possibility that different bacterial proteins are required for f2, MS2, and R17 RNA replication. It should be noted that in the case of f2, a differential effect on the in vivo synthesis of minus and progeny plus strands has already been described (23). This was shown in a class of f2 mutants having a temperature-sensitive replicase protein. When cells were infected with the mutants at the permissive temperature for 90 min and then shifted to 43 C, the synthesis of progeny RNA continued, although the production of minus strands was arrested. In addition, in the in vitro f2 replication system it was shown (N. V. Federoff, Ph.D thesis, The Rockefeller University, New York, N.Y., 1972) that f2 replicase complex had a higher affinity for f2 minus strands than for f2 plus strands.

Thus far no physiological differences among the group I phages, f2, MS2, and R17, have been described. Nevertheless, the present study reveals, for the first time, a difference in their replication process, which seems to be in extreme contrast with the very close similarity in their particle properties. However, the extreme similarity in the amino acid sequence of their coat proteins seems to be misleading for an anticipated equivalent resemblance at the nucleotide level. This assumption is supported by a comparison of 238 nucleotide positions of the coat cistrons of R17 and MS2 which revealed eight differences (26, 45). All but one of the base changes observed affected the third position of the degenerate codons and, therefore, appeared without expression on the amino acid sequence. In addition, an overall variation in the RNAs of group I phages was observed (28, 35); this was 2.8% for f2 versus MS2, as well as R17 versus MS2, and 3.4% for f2 versus R17. On the other hand, a comparison of the nontranslated regions revealed a far lower variability (3, 35). No difference was found for the 5' and 3' terminal sequences comprising a total of 181 nucleotide (3, 26). It seems that the genetic drift which exists in the cistronic regions of group I phage RNAs has a minimal effect with regards to protein structure; therefore, the derived mutations can be considered as neutral in many respects. However, the variations among these RNAs are probably considerably amplified in their spatial arrangements and, as a result, variations in their template recognition apparatus are expected. Recognition of the RNA template must involve more than just the primary structure of a limited region at its 3' end (16), and it seems likely that a region in the middle of the Qβ RNA molecules is involved in the template recognition of Qβ replicase, since the 3' terminal fragments of Qβ RNA are good templates only if they are no shorter than half the RNA molecules (38). According to the recently proposed model of Weissmann et al. (45), the specificity of the interaction between enzyme and template may not reside as much in the stringent of one or two localized features (primary or secondary structure) of the RNA as in the spatial relationship between two distant RNA regions which must interact simultaneously with two enzymatic sites. Concerning the elements in the replicase complex involved in the process of template recognition, recent experiments (22) indicate that in the case of Qβ replicase the RNA polymerizing activity of the enzyme resides in the phage-coded subunit II, whereas the host subunit III and IV appear to play an essential role in maintaining the active conformation of the replicase to recognize the template. The other bacterial proteins, subunit I of Qβ replicase, and the host factors (F1 and Fl) are exclusively needed for the replication of Qβ plus RNA strands, and not for any other template, and therefore presumably are involved in the recognition of replicase binding sites on the Qβ RNA strands (17, 19, 40). On the basis of these data we suggest that bacterial proteins play a central role in the template recognition process of Qβ, as well as in the same process of other RNA phages. The RNA-containing phages are extremely dependent on cellular components for their development because of their limited genetic content. It seems likely that in the process of RNA replication the phage provides only a core with RNA polymerizing activity and utilizes host proteins to recognize the RNA template either directly or indirectly. Based on the assumption that in the process of template recognition bacterial proteins and the tertiary structure of the RNA play an essential role, one would expect that different bacterial proteins might be used for this process by different RNA phages, as well as for the recognition of plus and minus RNA strands by the same phage. The possible role attributed
here to bacterial proteins in the RNA replication of the parasite may derive from their analogous function in the host of specific interaction with homologous RNA sequences or spatial arrangements of the binding sites.

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LITERATURE CITED


