ACTIVE FRAGMENTS OF A FILAMENTOUS BACTERIOPHAGE*

George Fareed, Karin A. Ippen, and Raymond C. Valentine
Department of Biochemistry
University of California
Berkeley, California 94720

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During the course of studies on the morphology of the flexible-thread-shaped bacteriophage f1 (Zinder et al., 1963) we observed that sonication of phage lysates sheared the phage particles into tiny subviral fragments about 1/6 the length of infectious particles (Stoeckenius, unpublished data). The broken particles were non-infectious (Marvin and Hoffman-Berling, 1963). We have reinvestigated the nature of these fragments particularly in view of recent findings that f1 attaches by one end to the sex hairs (F-pili) of male cells (Caro and Schnös, 1966; see also Tzagoloff and Pratt, 1964, and Ippen and Valentine, 1966). We have been particularly interested in whether the end fragments could still inject their nucleic acid into the male cell. In this communication we show that these small subviral fragments labeled with radiophosphorus (P^{32}) are apparently "active" and can adsorb and inject normally, although their cellular fate is not known. Using a type of dilution or competition experiment, it was found that the segments adsorb to cells as effectively as the intact particles. We presume that only the end segments are active though this has not been fully proven. The preparation and assay of active fragments are described.

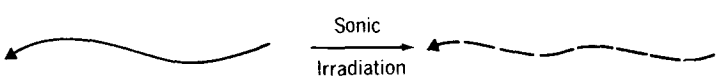

Fig. 1. Sonic irradiation of filamentous phage causes fragmentation of the particles into tiny nucleoprotein segments. The arrow at the tip is used to indicate the possible adsorption site.

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Results and Experimental

Radioactive fl. A highly purified, P^{32}-labeled stock of fl was prepared by infecting 20 ml of a male culture (2×10^8 cells/ml), grown in low phosphate medium (Valentine and Strand, 1965) containing 4 mc of radio-phosphorus, with 50 fl per cell. After 3 hours for production of phage, the lysate was centrifuged to remove cell debris and the phage precipitated from solution by adding 10.5 g solid $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ pellet of phage was collected by centrifugation, resuspended in water, and dialyzed for 3 hours to remove the $(NH_4)_2SO_4$ and residual radio-phosphorus. The dialyzed sample was centrifuged at 10,000 x G for 5 min to remove additional debris formed during dialysis. Solid CsCl (1.7 g/4 ml phage suspension) was next added to the dialyzed preparation and the phage banded by centrifuging for 24 hours at 37,000 rpm using the Spinco SW swinging bucket rotor. The phage band was collected from the tube by monitoring with a Geiger counter and centrifuged a second time in CsCl. The band from the second CsCl run was collected and dialyzed for 4 hours to remove CsCl. A normal preparation contained 2-4 $\times 10^{12}$ infective particles with a total activity of 2-3 $\times 10^7$ cpm. The concentrated radioactive phage was stored in the refrigerator and used for several weeks.

Shearing the Filamentous Phage by Sonication. Marvin and Hoffman-Berling (1963) originally found that the filamentous phage M13 (fl) was rapidly destroyed by sonication. They proposed that this thin, threadlike virus (8000 Å x 40 Å) was fragmented during sonication as shown schematically in Fig. 1. In agreement with their proposal we have viewed a sonicated preparation of fl in the electron microscope and found that the virus was shattered into tiny segments (Stoeckenius, unpublished micrographs). A second procedure - sucrose gradient analysis - was used to determine the relative size of the viral segments. This analysis is presented in Fig. 2. For this experiment, a sample of radioactive frag-

ments treated for 4 min with sonic irradiation was layered on a sucrose gradient (Fig. 2). As a marker, the gradient was assayed for the infectious particles remaining after sonication; the viable phage peak is indicated on the gradient. Using the infectious phage band as reference, it is obvious that after 4 min of sonication the majority of the radioactivity of the sample trails considerably behind (on the lighter side) of the whole phage, indicating that the virus filament has indeed been segmented.

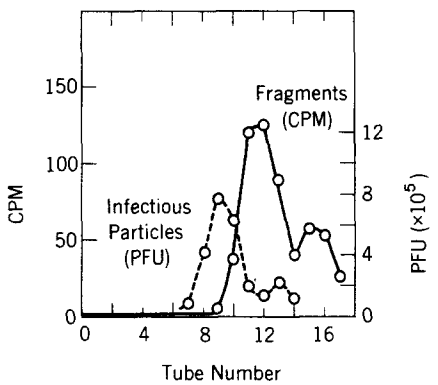

Fig. 2. Separation of fragments and whole phage on a sucrose gradient. A 0.2 ml sample of 4 min sonicated fragments was layered on a linear sucrose gradient (5-20%) and centrifuged for 6 hr at 37,000 rpm using a Spinco SW 39 swinging bucket rotor. Samples from the gradient were assayed for radioactivity and infectivity (PFU). Note that the bulk of the radioactivity (fragments) trails behind the infectivity peak.

We have not attempted to quantitatively evaluate the sedimentation constant of the fragments; intact phage banded at approximately 40s.

In order to fully rule out the possibility that whole phage was contributing to the activity of the fragment preparations a large-scale sucrose gradient was prepared in which all the samples of the gradient were analyzed for their "adsorption activity." This method is described below. Adsorption activity was found to follow the overall "CPM" profile on the gradient with no increased activity at the whole phage peak. This experiment also shows that fragments of different sizes are capable of adsorption.

The infectivity of phage f1 is also readily degraded by sonic irradiation, as shown in Fig. 3 (curve labeled PFU). Note in Fig. 3 that after 4 min sonication using a 10 KC sonic oscillator, 99.47% of the infectivity of an f1 preparation had been destroyed; after 8 min more than 99.99% of the infectivity of the suspension was destroyed. It seems probable that this destruction of infectivity is caused by the shearing of the filamentous phages into fragments.

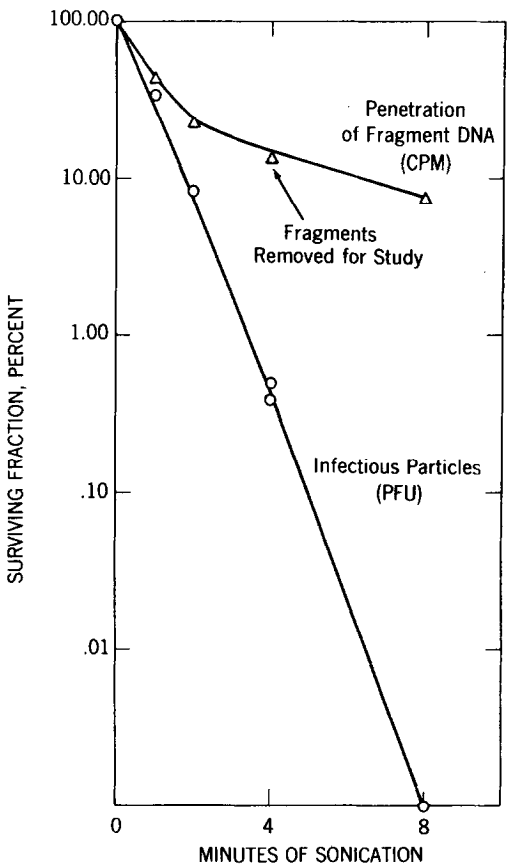

Fig. 3. Active fragments of bacteriophage f1 prepared by sonic irradiation (sonication). Radioactive phage sample (about 5 x 10^9 phage per ml in broth) treated at times indicated with sonic irradiation from a 10 KC sonic oscillator at maximum efficiency. See Fig. 4 for penetration assay.

Activity of Fragments. Since a radioactive phage preparation was used in Fig. 3, we next tested the sonicated samples to see if some of

the fragments (presumably the ends) still retained their ability to attach and inject their DNA into the male bacterium. The penetration activity of the sonicated fragments was measured by adsorbing the fragments to male bacteria at 37°C and measuring the penetration of radioactivity into the bacteria after blending the cells to remove particles which had not injected; this procedure is described in more detail below. As seen in Fig. 3 (penetration curve) the penetration activity dropped rapidly during the first minute of sonication and reached a plateau level after 4 minutes; during the 4-8 minute period only a small loss in penetration activity was observed. Note from the curves that the drop of infectious titer was linear during this time period (4-8 min). Even after 8 min sonication the fragments penetrated about 7% as much DNA as the untreated control. Since more than 99.99% of the infectivity was lost from the preparation at this time, it seems unlikely that the observed penetration could be coming from the whole phage remaining in the sonicated preparation. The activity of the preparation was about 1 cpm per 10^5 particles and at the most only a few cpm above background would be expected from whole phage. We conclude from this experiment that the subviral fragments which cannot be removed from the host cell by blending have in fact penetrated the host.

The adsorption and penetration properties of the fragments were studied further. Fig. 4a shows the rate of adsorption of the subviral particles to male cells. For this experiment the radioactive fragments sonicated for 4 min were adsorbed to male cells (10^9 cells/ml) at 0°C to prevent subsequent penetration of the DNA. At 0° Tzagoloff and Pratt (1964) observed that adsorption occurred at only a moderately reduced rate. The fragments adsorbed readily at this temperature (Fig. 4a) and reached a plateau after approximately 15 per cent (nonsonicated sample taken as 100 per cent) of the radioactivity of the preparation had adsorbed.

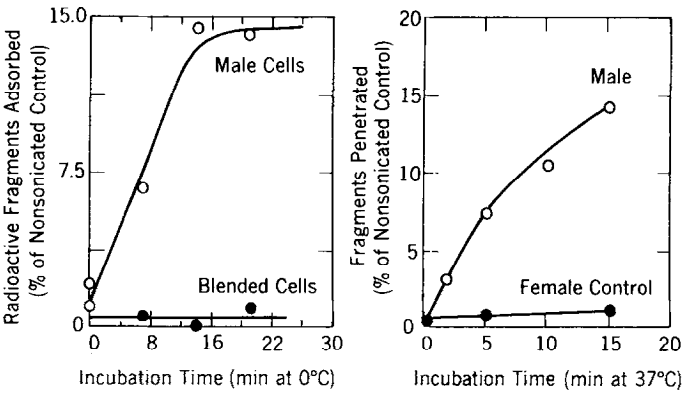

Fig. 4a. Adsorption of fragments and removal of adsorption sites by blending. See text for details of assay. Incubation was at 0°C. Phage sample was sonicated for 4 min. To remove F-pili the male culture was blended for 1 min using the Servall Omni-Mixer.

Fig. 4b. Active penetration of fragments into the male cells. Sonicated fragments (4 min) were added to male cells (5 x 10^8 cells/ml) (37°C) and 5 ml aliquots were removed at the times indicated and rapidly cooled to 0°C to stop further penetration. Phage fragments which had not injected were removed by blending; the cells containing radioactive DNA were collected by centrifugation and assayed for radioactivity.

In Fig. 4a fragments which were not able to adsorb to cells were removed during the centrifugation step of the adsorption assay. The use of radioactive phage to measure adsorption and penetration is described in detail in an earlier paper (Ippen and Valentine, 1966). We have also previously reported that blending of male cells to remove F-pili removed the f1 adsorption sites (Ippen and Valentine, 1966). As shown in the lower curve of Fig. 4a, blending drastically reduced the number of adsorption sites for the fragments as expected if F-pili were required. In Fig. 4b, penetration of fragments was followed as a function of time. A female culture served as a control for this experiment. Normally, a plateau level of penetration was observed using 4 min sonicated fragments after about 15% of the penetrable counts of the nonsonicated control had penetrated the cells.

It should be mentioned that although comparison of the absolute amount of radioactivity which adsorbs to that which penetrates has led us to suspect that nearly all the fragments which adsorb also eventually penetrate the cell, the differences in the assay procedures preclude any definite conclusions on this point. Mainly many fl-F-pili complexes because of their fragile nature probably break off the cells during the centrifugation procedure of the adsorption assay (Ippen and Valentine, 1966) and thus do not score in the assay.

Are Only the Phage Tips Active? It seemed reasonable to argue that only the viral end segments or "tips" were active in the adsorption and penetration assay. With this idea in mind, a type of competition experiment was carried out (Fig. 5) in which fragments from a nonradioactive preparation were used to compete with radioactive whole phage for pili adsorption sites. The aim of this experiment was to measure the number of active segments in the sonicated preparation. First, a "standard competition or dilution curve" was run, using nonradioactive whole particles (Fig. 5). An identical preparation was sonicated and assayed simultaneously for their ability to compete for adsorption sites with the radioactive virus. The active ends should fill the F-pili sites thus preventing radioactive particles from attaching. Such competition is evident from Fig. 5; note that the fragments appeared to have about as much "pili blocking power" as an equivalent amount of whole phage. It was puzzling at first why more PFU's of nonradioactive phage (crude lysate) than expected from calculations were needed to block adsorption of an equivalent number (PFU) of radioactive phage. This might be explained if the purified radioactive preparation contained a number of "dead" particles capable of adsorbing to pili, but not producing progeny (plaques).

Further analysis of the data from Fig. 5 leads to some other interesting conclusions. If it is assumed that fragments and whole phage adsorb to

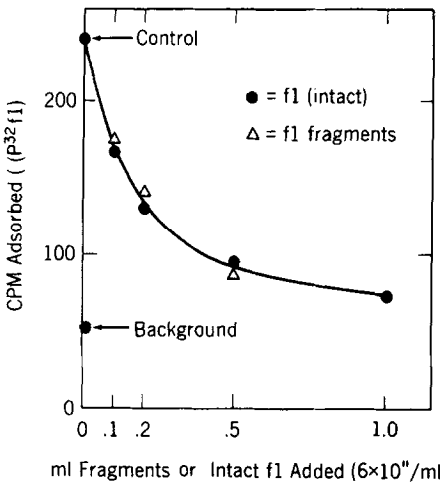

Fig. 5. Fragments of f1 effectively compete with intact phage for pili adsorption sites. Male cells (5 ml at 2 x 10^8 cells/ml) were infected at 0°C with about 2 x 10^{10} radioactive f1. Nonradioactive f1 or "cold" fragments were added immediately in the quantities shown. Phage adsorption was assayed as for Fig. 4. Note (for example) that 0.5 ml of fragments (8 min sonication) "competes" about as well as an exact quantity of untreated virus. Penetration was prevented by carrying out the reaction at 0°C.

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the pili at the same rate, the fact that no appreciable blocking power was lost or gained during sonication indicates that the original adsorption sites of the phage are left intact on the segments and that only these, and no newly generated ends (see Fig. 1), are capable of adsorption. (It should be noted again that the experiment was run at 0°C, therefore no penetration is occurring which could free the pili site for a new fragment.) In addition, although it has not yet been shown definitively whether the intact phage is capable of adsorbing at only one or both of its ends, our data seem to suggest that only one active end per phage particle is present.

If both ends were active, breaking the phage filament should have yielded two independent adsorbing segments per original phage particle. Consequently, unless one makes the unlikely assumption that most of the whole phage were actually adsorbed to two separate pili in the cell

cultures employed (Caro and Schnös did not report seeing any such pili-f1-pili complexes), sonication would have given the phage lysate up to twice the blocking power of the original lysate. Thus, although further studies are necessary to establish this fact, it seems probable to us that the whole phage contains only one adsorbing end, and that after sonication, it is only the fragment from this end which adsorbs. In other words, f1 may have a left and right end.

Discussion

Perhaps it is not surprising that the male cell is able to concentrate DNA from the fragments of f1. It has been shown previously that RNA from phage f2 is transported by the "F-pili transport mechanism" (Ippen and Valentine, 1966). Similarly, Notani and Zinder (1964) were able to infect cells with unstable "diploid" phages - two phages whose ends apparently had been joined through protein-protein interaction of the capsids. Caro and Schnös (1966) have also observed what appear to be "triploids" - three phages joined together. It is not known whether these triploids are able to penetrate cells. These experiments indicate that the mechanism for nucleic acid penetration by the male cell is not entirely specific with regard to the forms of nucleic acid which can be transported.

The size of the nucleic acid fragments which enter the cell is of considerable interest. Earlier micrographs (Stoeckenius, unpublished data) indicated that the phage fragments were about 1/6 the size of normal phage. It was also of interest that few intact particles could be seen after sonication. If one assumes that only the ends are penetrating and that the DNA forms its loop in the end fragments (the DNA is thought to be a closed circle in its capsid with a coding capacity of 6-10 genes) then the linear segment of DNA entering the male cell might be as small as 1-2 cistrons. Studies on the cellular fate and function of these "1-cistron" viral segments are in progress.

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