*CHROMOSOME STRUCTURE IN PHAGE T4, II. TERMINAL REDUNDANCY AND HETEROZYGOSIS**

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Communicated by A. D. Hershey, September 15, 1965

In a previous communication[1] it was suggested that the chromosome of each particle of phage T4 is terminally redundant, and that after several rounds of replication (following infection), progeny chromosomes arise which have circularly permuted genetic sequences. Among the members of a population of phage particles, the ends of the chromosomes would be randomly distributed over the genome, and a phage particle carrying different alleles at one of its redundant loci would be heterozygous.

Nomura and Benzer[2] have reported that heterozygotes for *r*II deletion mutants (from mixed infections with r^+ phage) occur with about a third of the frequency of those for *r*II point mutants. It thus seemed likely that heterozygosity in phage T4 is of at least two kinds. One kind may be the result of heterozygosity within the molecule, as was first suggested by Levinthal,[3] whereas another kind may be due to the terminal redundancy described above. Deletion mutants may be unable to form heterozygotes of the internal kind since these would involve violations of duplex complementarity (regardless of their precise molecular structure), whereas point mutants may form heterozygotes of both kinds.

The chromosome of a phage particle will be terminally redundant and heterozygous for marker *a* provided that (1) the chromosome terminates near marker *a* and includes marker *a*, and (2) the last recombinational event, occurring somewhere within the chromosome, has been such that the other end of the chromosome contains marker a^+. *Terminal-redundancy heterozygotes are formed and lost by the process of recombination; their frequency (as a function of time after infection) will depend on the rate of recombination but not on the rate of replication.* In a pool of vegetative chromosomes, the frequency of terminally redundant heterozygotes would be expected to change in time as does the frequency of recombination for very distantly linked markers; it would be expected to increase until an equilibrium value was reached and to remain constant after that. Because of the high frequency of recombination in phage T4, one would expect that the equilibrium value would be reached very early during the latent period.

Internal heterozygotes are most probably formed by an event that results in the recombination of markers on either side of the heterozygous region;[3] thus, they may well represent the primary products of recombination. They would be expected to disappear upon the semiconservative replication of the chromosome bearing them. The chromosome of a phage particle will be internally heterozygous for marker *a* provided that (1) a recombinational event leading to internal heterozygosis will have occurred at the site of marker *a*, and (2) the chromosome is incorporated into a mature particle before it undergoes replication. *Internal heterozygotes are formed by recombination and are lost by replication: their frequency (as a function of*

time after infection) will depend both on the rate of recombination and on the rate of replication.

The frequency of heterozygotes, as a function of time after infection, has been observed to remain constant.[4] In a standard cross, the formation and disappearance of heterozygotes is at equilibrium throughout the period during which phage are withdrawn from the vegetative pool. If the rate of replication could be substantially decreased during this period, the frequency of internal heterozygotes would be expected to rise.

The thymidine analogue fluorodeoxyuridine (FUDR) inhibits DNA synthesis in phage-infected bacteria[5] but does not interfere with recombination.[6] It would be expected that in phage-infected, FUDR-treated bacteria, the frequency of internal heterozygotes would increase as a function of time, whereas the frequency of terminal-redundancy heterozygotes would remain constant. A test of this prediction forms the substance of this communication.

Materials and Methods.—The phage strains used were T4B standard type and mutants *r*607, *r*287, *r*205, *r*1364, *r*W8-33, *r*H23, *r*168 (all obtained from Dr. S. Benzer); T4D standard type and mutants *ts*N30 and *r*EDb42 (obtained from Dr. R. S. Edgar); and T4 h^{2+} (obtained from Dr. G. Stent). Stent's strain was derived by him by crossing T4 and T2, crossing a progeny strain with the T2 host range to T4, and repeating the process a number of times. The h^{2+} locus of Stent's strain was incorporated by us into the appropriate T4B and T4D strains by exposing T4 h^{2+} phage to a dose of ultraviolet light sufficient to produce about 20 phage lethal hits, crossing the UV-treated T4 h^{2+} with the appropriate phage strain and selecting a (rare) progeny strain with the h^{2+} host range. The standard-type T4 strain is designated h^{4+}.

The bacterial strains used were *Escherichia coli* B, B/2, B/4, S/6, and K12 112-12(λh), hereafter called K.

Media: Broth: H_2O, 1 liter; bacto-tryptone, 10 gm; NaCl, 5 gm. Tryptone bottom agar: broth with 1.1% bacto-agar. Tryptone top agar: broth with 0.7% bacto-agar. EHA bottom agar: tryptone bottom agar, 1 liter; sodium citrate·$2H_2O$, 1.1 gm; glucose, 1.3 gm. EHA top agar: tryptone top agar, 1 liter; sodium citrate·$2H_2O$, 1.1 gm; glucose, 3.0 gm. M-9: H_2O, 1 liter; Na_2HPO_4, 7 gm; KH_2PO_4, 3 gm; NH_4Cl, 1 gm; supplemented after autoclaving with glucose, 4 gm; $MgSO_4$, 10^{-3} *M*.

Fluorodeoxyuridine was a gift from Dr. R. Duschinsky of Hoffman-LaRoche, Inc.

Phage stocks were prepared by stabbing a 4- to 5-hr plaque with a needle and rinsing the needle into 20 ml of B cells grown to a concentration of about 10^8 cells per ml in aerated M-9 at 37°. The stocks were lysed by the addition of chloroform after about 5 hr of aeration. Stocks of *ts* mutants were prepared at 30°. DNase was added to the lysates, and the lysates were centrifuged at low speed to remove bacterial debris. Stocks of h^{2+} phage were incubated at 65° for $2^1/_2$ min immediately after lysis and centrifugation.[7]

Assay of phage: For platings on K or on B, the bacteria were grown to a concentration of 2×10^8 cells per ml in aerated broth at 37°, and 0.5-ml amounts were used per 2.5 ml of tryptone top agar to overlay plates containing 36 ml of tryptone bottom agar.

For platings on B/2 + B/4, appropriate amounts of phage were added to B grown to a concentration of 2×10^8 cells per ml in aerated broth at 37°. After 5 min of incubation at 37°, one part of the phage-B mixture was added to two parts of an equal mixture of B/2 and B/4, each grown to a concentration of 2×10^8 per ml in aerated broth at 37°. One part of the final mixture was added to five parts of melted tryptone top agar at 45°, and 3.0-ml samples were immediately distributed to as many plates containing 36 ml of bottom tryptone agar as were necessary.

For platings on S/6, the bacteria were grown to a concentration of 2×10^8 cells per ml in aerated broth at 37°, chilled, and concentrated tenfold by centrifugation. Two drops of the concentrated bacteria were added to 2 ml of EHA top agar, 0.05 ml of the appropriate dilution of phage was added, and the mixtures were poured on plates containing 36 ml EHA bottom agar. In some cases phage were first adsorbed to concentrated B and were then diluted and plated on S/6.

Results.—The frequency of heterozygotes as a function of time: The frequency of

heterozygotes for point and deletion rII mutants was measured at various times after infection in the presence of FUDR.

Since little DNA synthesis takes place in the presence of FUDR, the removal, through maturation, of the few DNA molecules in the vegetative pool needs to be prevented. This can be accomplished by the addition of chloramphenicol (CAM) to the infected bacteria at about 9 min after infection. The chloramphenicol is removed at various times after infection, and the infected bacteria remain in the presence of FUDR for (usually) another 60 min and are then lysed by the addition of chloroform. The infected bacteria are thus always in the presence of FUDR.

The frequency of heterozygotes as a function of time is illustrated in Figure 1. As expected, the frequency for point rII mutants increases, that for deletion rII mutants stays constant.

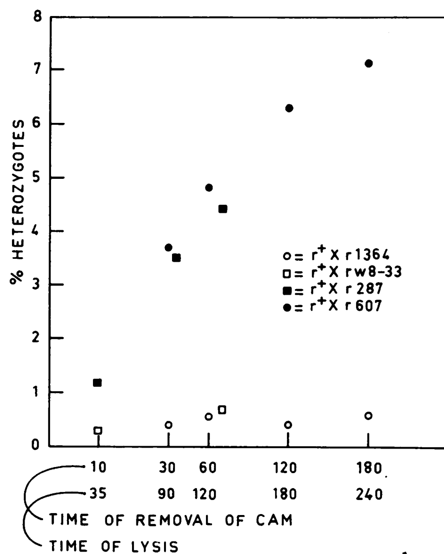

Fig. 1.—The frequency of heterozygotes after various periods of incubation in FUDR. Bacteria of strain B were grown to a concentration of 10^8 per ml in aerated M-9 supplemented with 0.5% bacto-casamino-acids, at 37°. To these cultures 20 μg of tryptophane per ml, 4×10^{-5} *M* FUDR, and 2×10^{-4} *M* uracil were added just before infection with an average of five phage particles of each type per bacterium. Nine min after infection, 250 μg of chloramphenicol per ml were added to the cultures, and at various times after that, aliquots of the cultures were chilled and washed twice by centrifugation, both the first and the second resuspension being with M-9 containing bacto-casamino-acids, uracil, and FUDR, but no chloramphenicol. The *time of removal of chloramphenicol* refers to the time, in minutes, after infection at which the cultures were chilled, before centrifugation. The cultures were then further incubated with aeration at 37°, and were subsequently lysed by the addition of chloroform, the *time of lysis* indicating the time in minutes after infection (omitting the time required for centrifugation). The lysates were plated on S/6, and the frequency of mottled plaques was determined after overnight incubation.

The frequency of recombinants as a function of time: The frequency of recombinants is expected to increase in the presence of FUDR and chloramphenicol in the same way as did the frequency of heterozygotes. That this is so is shown by the results presented in Figure 2.

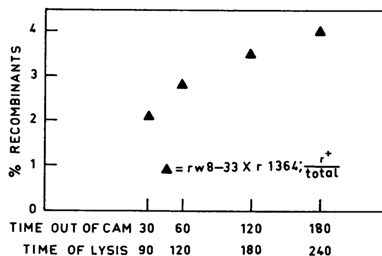

Fig. 2.—The frequency of recombinants after various periods of incubation in FUDR. The procedure used was exactly as described for Fig. 1, except that platings were on B and on K. Plaques appearing on plates containing K represent r^+; those appearing on B represent all types.

The frequency of heterozygotes in the presence and absence of FUDR: The frequency of heterozygotes formed by a number of different deletion rII mutants and by point rII mutants was compared. In each case FUDR was added near the time of infection, chloramphenicol was added 9 min after infection and removed at about 120 min after infection, and the infected

bacteria were lysed about 60 min after the removal of chloramphenicol. Control cultures were prepared in the absence of FUDR. The frequency of heterozygotes increased in the presence of FUDR for each of the point mutants examined, but remained the same as the control frequency for the deletion mutants (Table 1).

TABLE 1

FREQUENCY OF r/r^+ HETEROZYGOTES IN THE PRESENCE AND ABSENCE OF FUDR

Nature of r mutant	Infecting phages	Mottled Plaques in the Absence of FUDR: Per cent	Number counted	Mottled Plaques in the Presence of FUDR: Per cent	Number counted
Point	$r607 \times r^+$	1.0	45	7.1	142
	$r287 \times r^+$	0.7	42	5.9	29
	$r205 \times r^+$	0.9	45	3.0	91
Deletion	$r1364 \times r^+$	0.5	24	0.6	44
	$rW8\text{-}33 \times r^+$	0.4	34	0.4	31
	$rH23 \times r^+$	0.5	42	0.4	34

Bacteria of strain B were grown to a concentration of 10^8 per ml, in aerated M-9 supplemented with 0.5% bacto-casamino-acids, at 37°; 20 μg of tryptophane per ml were added to the bacteria just before they were to be infected. For mixed infections in the absence of FUDR, the bacteria were then infected with an average of about five phage particles of each type per cell, incubated with aeration at 37°, and lysed by the addition of chloroform at about 25 min after infection. For mixed infections in the presence of FUDR, 4×10^{-6} M FUDR and 2×10^{-4} M uracil were added just before infection, the bacteria were infected with an average of about five phage particles of each type per cell, and 250 μg chloramphenicol per ml were added 9 min later. After 120 min of incubation, with aeration, at 37°, the bacteria were washed twice by centrifugation, both the first and second resuspension being with M-9 containing bacto-casamino-acids, uracil, and FUDR, but no chloramphenicol. The infected bacteria were lysed by the addition of chloroform after a further 60 min of incubation with aeration. The lysates were in all cases treated with DNase and then centrifuged at low speed to remove bacterial debris. The lysates were plated on S/6, and the frequency of mottled plaques was determined after overnight incubation.

The behavior of h^{2+}/h^{4+} heterozygotes: Genetic markers (other than rII deletions) that would not form internal heterozygotes seemed very desirable for a number of experiments described below and elsewhere. The host-range difference between phages T2 and T4 is under control of allelic markers; no recombinants possessing a combination of host ranges have been observed.[8] That the host-range difference might be due to "complex" genetic changes was suggested by the observations that a change from T2-like to T4-like host range (or vice versa) could not be induced by treatment with mutagens,[9] and that the frequency of h^{2+}/h^{4+} heterozygotes was low[8] and of the order observed by Nomura and Benzer[2] for deletion mutants.

It seemed important to determine whether the h^{2+}/h^{4+} heterozygotes were similar to those formed by deletion mutants. The frequency of heterozygotes among the progeny phage produced in bacteria mixedly infected with h^{2+} and h^{4+} phage and incubated with and without FUDR was therefore measured. As indicated in Table 2, the frequency was similar in the presence and absence of FUDR, indicating that h^{2+}/h^{4+} heterozygotes behave in the manner expected for terminal-redundancy heterozygotes.[10]

Double heterozygosis: Plaques that contain two types of phage particles could be formed either by a heterozygous phage particle or else by a clump of several parti-

TABLE 2

FREQUENCY OF h^{2+}/h^{4+} HETEROZYGOTES IN THE PRESENCE AND ABSENCE OF FUDR

Infecting phages	Clear Plaques in the Absence of FUDR: Per cent	Number counted	Clear Plaques in the Presence of FUDR: Per cent	Number counted
$rH23\ h^{2+} \times rH23\ h^{4+}$	0.85	75	0.85	52

The procedure was exactly as described for Table 1, except that the lysates were plated on B/2 + B/4 and the frequency of clear plaques was determined after overnight incubation. Each plate contained fewer than 75 plaques.

cles. In order to determine whether clumps of particles are responsible for the formation of plaques containing both h^{2+} and h^{4+} particles (h^{2+}/h^{4+} plaques), a cross of $h^{2+}r^+ \times h^{4+}$ $r168$ was performed. The marker $r168$ is a small deletion that is about 20 units distant from h^{2+}. Among the progeny of this cross, about 30 per cent of h^{2+}/h^{4+} plaques were found to contain both r and r^+ phage (Table 3)

TABLE 3

ANALYSIS OF h^{2+}/h^{4+} PROGENY PLAQUES AFTER FRACTIONATION THROUGH SEDIMENTATION IN A DEUTERIUM GRADIENT

Fraction	Total number of plaques examined	Number of h^{2+}/h^{4+} plaques found	Frequency of h^{2+}/h^{4+} plaques	Fraction of h^{2+}/h^{4+} plaques that contained both r and r^+ phage
Peak drop	4.6×10^4	90	2.0×10^{-3}	0.07
1	2.3×10^3	3	—	0
2	2.9×10^3	10	3.4×10^{-3}	0.10
3	2.1×10^3	15	6.7×10^{-3}	0.32
4	2.5×10^3	50	2.0×10^{-2}	0.72
Unfractionated lysate	2.0×10^4	53	2.7×10^{-3}	0.30

Fractions from the deuterium-gradient sedimentation of the progeny of a cross of h^{2+} r^+ $\times$ h^{4+} $r168$ described in Fig. 3 were plated on B/2 + B/4; each plate contained fewer than 40 plaques. Clear plaques were picked and replated on B/2 + B/4 to test for the presence of h^{2+} and h^{4+} phage (and to exclude the rare plaques containing, instead, h^{2+} and h^2 phage). The clear plaques were also plated on B and were scored with respect to whether they contained r, r^+, or both r and r^+ phage.

and could thus have been formed by clumps. As will be shown, the actual fraction of spurious heterozygotes is somewhat less than 30 per cent, too low to invalidate the experiment with FUDR presented above. For other purposes, however, it was necessary to isolate the true heterozygotes.

Since it was expected that clumps would sediment more rapidly than single phage particles, the progeny of the cross were centrifuged in a deuterium oxide gradient[11] and fractions were collected from the bottom of the centrifuge tube (Fig. 3). The frequency of h^{2+}/h^{4+} plaques and the proportion of these that contained both r and r^+ phage were measured for the peak drop and for the more rapidly moving fractions. As shown in Table 3, the frequency of h^{2+}/h^{4+} plaques was con-

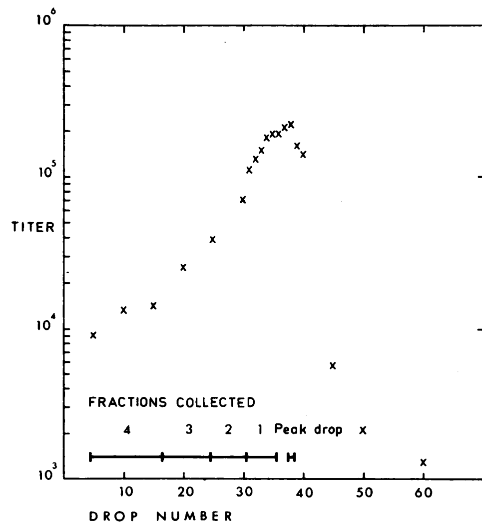

FIG. 3.—The fractionation of progeny phage of a cross through sedimentation in a deuterium gradient. Bacteria of strain B were grown to a concentration of 10^8 per ml in aerated broth at 37°, chilled, and concentrated by centrifugation. The bacteria were infected at a concentration of 10^9 per ml with an average of six phage particles each of h^{2+} r^+ and h^{4+} $r168$. After 2 min incubation at 37°, they were diluted in broth at 37° and were incubated at that temperature; 30 min after infection, the bacteria were lysed by the addition of chloroform.

A continuous gradient of D_2O was produced in a centrifuge tube by mixing broth prepared with D_2O and broth prepared with H_2O. An aliquot of broth containing the progeny of the cross was delivered to the surface of the gradient and the tube was centrifuged for 15 min at 20,000 rpm in the SW39 head of a Spinco centrifuge. After centrifugation, a hole was punched in the bottom of the tube, and individual drops were collected in tubes containing 0.5 ml of broth. The contents of tubes were pooled as indicated in the figure to yield fractions 1–4.

siderably greater, and a greater proportion of them contained both r and r^+ phage in the more rapidly moving fractions than in the peak drop. In the most rapidly moving fraction examined, 72 per cent of the h^{2+}/h^{4+} plaques contained both r and r^+ particles, whereas in the peak drop, only 7 per cent of the h^{2+}/h^{4+} plaques contained both r and r^+ particles.

The fast-moving fraction was heated for a length of time sufficient to inactivate 90 per cent of the phage particles. This treatment drastically reduced the frequency of apparent heterozygotes, an indication that at least a large proportion of them were clumps. Thus the rapidly sedimenting particles forming mixed plaques are mainly clumps, justifying the assumption that those sedimenting normally are authentic heterozygotes. If so, the data of Table 3 show that about 7 per cent of the authentic h^{2+}/h^{4+} heterozygotes are also heterozygous for the r marker, and that only about 25 per cent of the particles forming mixed plaques are clumps.

Recombination for neighboring markers among heterozygotes: Terminally redundant heterozygotes ought to be recombinant with respect to neighboring markers. In a cross of $a\ b\ c \times a^+b^+c^+$, for instance, terminal-redundancy heterozygotes that are b/b^+ would be expected to have either of two possible arrangements of markers:

$$\underline{b\ c \qquad\qquad a^+\ b^+} \quad \text{or} \quad \underline{b^+\ c^+ \qquad\qquad a\ b},$$

both arrangements being recombinant with respect to the markers a and c.

In order to determine whether particles that are terminal-redundancy heterozygotes for a particular marker are recombinant for neighboring markers, a cross of rEDb42 h^{4+} tsN30 $\times$ r^+h^{2+} ts^+ was performed. The r and ts markers are on opposite sides of the host-range marker and yield about 20 and 17 per cent recombinants, respectively, in crosses with the host-range marker. The progeny from the cross were centrifuged in a deuterium gradient and the contents of the peak drop were plated at 43° on B/2 + B/4. Only ts^+ phage (or phage heterozygous for ts/ts^+) produce plaques under these conditions. Among the plaques appearing at 43°, h^{2+}/h^{4+} heterozygotes were scored with respect to the r marker. A majority of the h^{2+}/h^{4+} heterozygotes that were ts^+ were r and thus recombinant, in contrast to the phage in the total population (Table 4). This cross was repeated with various other combinations of these same markers in order to show that the result did not depend on the particular alleles used (Table 4). We may thus conclude that h^{2+}/h^{4+} heterozygotes are recombinant with respect to neighboring markers

TABLE 4

ASSORTMENT OF OUTSIDE MARKERS AMONG h^{2+}/h^{4+} HETEROZYGOTES

Cross	Type of plaque examined	Number of plaques examined	Fraction of Plaques That Contained r	r^+	r and r^+
$r\ h^{4+}\ ts \times r^+\ h^{2+}\ ts^+$	Clear ts^+	85	0.59	0.18	0.23
	All ts^+	50	0.20	0.80	0
$r^+\ h^{4+}\ ts \times r\ h^{2+}\ ts^+$	Clear ts^+	145	0.25	0.49	0.26
	All ts^+	50	0.80	0.20	0
$r^+\ h^{4+}\ ts^+ \times r\ h^{2+}\ ts$	Clear ts^+	28	0.68	0.14	0.18
	All ts^+	49	0.37	0.63	0

Bacteria of strain B were grown to a concentration of 10^8 per ml in aerated broth at 37°, chilled, and concentrated by centrifugation. The bacteria were infected at a concentration of 10^9 per ml with an average of six phage particles of each parental type. After 2 min at 30°, they were diluted into broth at 30° and incubated at that temperature; 55 min after infection, the bacteria were lysed by the addition of chloroform. An aliquot of the progeny of each cross was centrifuged in a deuterium gradient as described in Fig. 3. The peak drop was plated on B/2 + B/4 and the plates were incubated at 43°. At this temperature, only plaques containing ts^+ phage are formed. Each plate contained fewer than 40 plaques. Clear plaques or else a random sample of all the plaques present were picked and analyzed as described in Table 3.

as would be expected on the basis of the terminal-redundancy model. A similar result had been obtained previously by Doermann and Boehner,[12] who examined heterozygotes formed by an *r*-deletion mutant. In our crosses an appreciable fraction of the h^{2+}/h^{4+} heterozygotes were simultaneously heterozygous for r/r^+ even though clumps had presumably been eliminated by the centrifugal fractionation.

Discussion.—The experiments reported here demonstrate the existence of two types of heterozygotes and thus confirm observations of others.[2] Heterozygotes of one type do not accumulate under conditions of limited DNA synthesis, and the behavior of this type of heterozygote confirms an important prediction of the terminal-redundancy model for the chromosome of phage T4.

An alternate model for this type of heterozygote may be considered: phage particles could consist of a whole chromosome and an additional small piece of a chromosome. This model is excluded by the observation presented here that h^{2+}/h^{4+} heterozygotes are recombinant for neighboring markers, and by the results of others[12] that deletion-mutant heterozygotes are recombinant for neighboring markers.

Summary.—Point-mutant heterozygotes increase in frequency during phage growth under conditions of limited DNA synthesis but deletion-mutant heterozygotes do not. Heterozygotes for the h^{2+}/h^{4+} marker behave as deletion-mutant heterozygotes do and are recombinant with respect to neighboring markers. These observations support the terminal-redundancy model for the chromosome of phage T4.

The authors are grateful to Dr. R. Duschinsky of Hoffmann-LaRoche, Inc., for a generous gift of fluorodeoxyuridine.

* This investigation was supported by research grants to G. Streisinger from the National Science Foundation (G-14055) and the National Institute of Allergy and Infectious Diseases (E-3892). Part of this investigation was carried out at the Biological Laboratory in Cold Spring Harbor, New York, during the summer of 1962. The authors are grateful for the hospitality exhibited by the staff of the Laboratory.

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[10] The frequency of h^{2+}/h^{4+} heterozygotes in this experiment is high owing to the presence of the long deletion *r*H23 in both parental stocks of the cross. The influence of deletions on the frequency of terminal-redundancy heterozygotes will be described in a subsequent communication.

[11] We are grateful to Professor Sidney Brenner, who suggested deuterium oxide as a convenient medium for this type of centrifugation.

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