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Regulation of imm Gene Expression in Bacteriophage T⁴-infected
Cells

by Masuo Yutsudo

ABSTRACT

Three polypeptides (imm-a, imm-b, and imm-c) which are not induced by an immunity mutant T4Dimm2 were identified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Their molecular weights were 77,000, 45,000, and 33,000, respectively. These polypeptides exhibited the similar kinetic pattern of synthesis. One other polypeptide (imm-d; molecular weight, 72,000) which was considered to be a fragment of imm-a appeared in the T4Dimm2-infected cells.

In a few minutes after infection, the primary phage established the system that inhibited imm gene expression of superinfecting phage. This was shown by measuring both the phenotypic expression of immunity and the synthesis of imm gene polypeptides. This shutoff was caused by phage-directed protein(s). Although imm gene belongs to the immediate-early class (Yutsudo and Okamoto, 1973), this shutoff did not extend to other immediate-early or delayed-early genes, since the expression of other early genes such as genes s, 30, and 33 was not affected by primary infection.

INTRODUCTION

During an early stage of phage T₄-infection, only a part of phage DNA is first transcribed into mRNA by host RNA polymerase immediately after its penetration into a host cell. This class of mRNA, so-called immediate-early mRNA, is synthesized even in the presence of inhibitors of protein synthesis (Grasso and Buchanan, 1969; Salser et al., 1970). Thereafter, follows delayed-early mRNA synthesis, for which a T₄-specific protein (or protein synthesis) is required (Grasso and Buchanan, 1969; Salser et al., 1970). Another class of prereplicative mRNA, called quasi-late mRNA, also begins to be produced at about the same time as delayed-early mRNA (Salser et al., 1970; O'Farrell and Gold, 1973b, 1973c).

Concerning the transcriptional change from the immediate-early to the delayed-early class, two hypotheses have been presented, i. e., "new promotor" theory and "elongation" or "anti-termination" theory. The former requires the participation of new promoters for delayed-early transcription. Phage-induced protein(s) causes some alteration of transcriptional machinery through which new promoters are recognized (Travers, 1969, 1970). The latter hypothesis does not need to introduce new promoters. In this case, delayed-early mRNA elongates from a distal end of immediate-early mRNA (Milanesi et al., 1969; Milanesi et al., 1970; Black and Gold, 1971;

Brody et al., 1971).

The fact that protein synthesis is indispensable for switching on delayed-early transcription suggests the existence of a factor responsible for such regulation. Travers (1969, 1970) has reported that T4 sigma factor stimulates the synthesis of delayed-early mRNA. However, this factor does not appear until 5 min after infection at 30°, whereas delayed-early mRNA synthesis is switched on at about 2 min after infection (Grasso and Buchanan, 1969; Salser et al., 1970). Recently, it has been reported by Daegelen and Brody (1976) that rIIA (delayed-early) and rIIB (quasi-late) RNA synthesis is controlled by a diffusible product.

However, according to the elongation theory, it is not always necessary to propose the participation of the factor. The lack of delayed-early mRNA in the absence of protein synthesis could be interpreted without any factor, if one assumes that synthesis of delayed-early mRNA is coupled with protein synthesis (Black and Gold, 1971).

In order to elucidate the regulatory mechanism of early transcription in phage T4, we studied early gene expression of superinfecting phage. In molecular genetics, superinfection has often been used to introduce a specific gene into host cells previously infected with a phage mutant defective in the gene. However, in the case of T-even phage, superinfecting phage DNA can not enter cell cytoplasm, and remains

within the cell envelope, presumably as a result of changes in the cell envelope induced by primary phage (Anderson and Eigner, 1971a, 1971b). This phenomenon is called exclusion.

The exclusion is considered to be brought about by phage-directed protein(s) judging from the following observations: (i) Primary infection with phage ghosts does not cause superinfection breakdown [infected cells acquire the ability, a few minutes after infection, to break down superinfecting phage DNA into acid soluble fragments (Graham, 1953)], (ii) chloramphenicol or rifampin inhibits the development of exclusion (Anderson and Eigner, 1971a), and (iii) UV-ray irradiation of cells does not affect it (our unpublished data). The synthesis of this protein is directed by T4 immediate-early mRNA (Yutsudo and Okamoto, 1973). Further, it has been reported that T4 mutant of immunity (imm) gene which is thought to code this protein does not bring about the exclusion (Vallée and Cornett, 1972; Childs, 1973).

In this paper, the regulatory mechanism of early gene expression of superinfecting phage T4 is studied by the use of a phage mutant T4Dimm2 which lacks the ability to exclude superinfecting phage.

The results were as follows. First, we identified T4D-induced three polypeptides which were not detected in T4imm2-infected cells by SDS-polyacrylamide gel electrophoresis. Kinetic studies showed the similar behavior of synthesis of

these polypeptides. Further, we showed that in a few minutes after primary infection, the infected cells became to inhibit imm gene expression of superinfecting phage. However, other early genes such as genes s, 30, and 33 of superinfecting phage were expressed even when cells were superinfected at long intervals after primary infection. We also showed the participation of phage-directed protein(s) in the shutoff. In DISCUSSION, we will propose postulates which may account for this shutoff.

MATERIALS AND METHODS

Bacteria. Escherichia coli B (su^-) and CR63 (su^+_{UAG}) were kind gifts from Dr. K. Okamoto.

Bacteriophages. T4D, T4Dimm2 (derived from T4DamE142), T4DamH39(30), T4DamN134(33), T4DamB22(43), T4Dimm2-tsL13(42), and T4Dimm2-s2 were kindly provided by Dr. K. Okamoto. T4Dimm2-tsL13-amH39 and T4Dimm2-tsL13-amN134 were constructed as described below.

Media. Cells were cultured in medium E (Vogel and Bonner, 1956) supplemented with glucose (0.2%) and casamino acid (0.1%). Phage assay was performed on the plate of agar (1%)-T4 medium (Tomizawa and Anraku, 1964).

Chemicals. Sodium dodecyl sulfate (sequanal grade) was purchased from Pierce Chemical Co., U.S.A., and other compounds required for SDS-polyacrylamide gel electrophoresis from Wako Pure Chemical Industries, Ltd., Japan. [^{35}S]-methionine (406 Ci/mmol), [^{14}C]amino-acid mixture (54 mCi/mAtom carbon), [^{14}C]uridine (500 mCi/mmol), and [3H]uridine (29 Ci/mmol) were products of The Radiochemical Center, Amersham, England.

Construction of T4Dimm2-tsL13-amH39 and T4Dimm2-tsL13-amN134. E. coli CR63 was infected simultaneously with two kinds of phage, T4Dimm2-tsL13 and T4 amber mutant (T4DamH39 or T4DamN134), and incubated 120 min at 30°. The lysate was

plated on E. coli CR63 at 30°. Each plaque formed was then tested on E. coli B at 30° and on E. coli CR63 at 42°. Under these conditions, the phage desired would not be able to make plaques, since imm gene is just adjacent to gene 42 (tsL13), namely, it is expected that the phage which has a temperature sensitive (ts) mutation would also possess a mutation of imm gene. The immunity of phage which was chosen by screening test described above was checked by the resistance of phage-infected cells against ghost infection.

SDS-polyacrylamide gel electrophoresis. Slab gel electrophoresis was carried out principally according to the procedures described by Maizel (1971) and by O'Farrell and Gold (1973a).

Radioactive protein samples were prepared as follows. After preincubation of UV-ray irradiated E. coli B in medium E containing glucose (0.2%) for 10 min at 30°, the cells were infected with phage at a multiplicity of 6, and labeled with radioactive amino acid during appropriate periods as indicated in the legends to Figs. 1, 2, and 4. The labeling was terminated by addition of 10% trichloroacetic acid (TCA). The precipitate was washed with 5% TCA (twice) and with acetone, and dissolved in sample buffer containing 0.0495M Tris-HCl, pH6.8, 1% SDS, 1% β-mercaptoethanol, 10% glycerol, and phenol red as a tracking dye. The slab gel was composed of a 10% separating gel and a 4% stacking gel (130 x 130 x 1mm), and it was run for 4-

4.5 hr at constant voltage (100V). After electrophoresis the proteins were fixed and stained with 0.2% Coomassie Brilliant Blue in 50% methyl alcohol and 10% acetic acid. The destained gels were dried and exposed on X-ray films (Kodak RP-R54).

Standard markers for estimation of molecular weight, [^{14}C]-labeled Adenovirus type 2 virions, were a kind gift from Dr. H. Yasue.

Measurement of immunity. The degree of immunity (or exclusion) can be estimated by the incorporation of radioactive leucine (Vallée et al., 1972) or uridine (Okamoto, 1973) into the cells superinfected with T4 ghosts. In this paper, we used only [^3H]uridine to measure it.

Ten minutes after infection at 30° (or after second infection if cells were superinfected), phage development was stopped by the addition of chloramphenicol (125 $\mu\text{g/ml}$). Then, at 25°, a tube containing ghosts or medium (without ghosts) was preincubated for 5 min, followed by the addition of the phage-infected cells prepared as above, and incubated for further 5 min. [^3H]uridine (29 Ci/mmol, 0.5 $\mu\text{Ci}/150 \mu\text{l}$ culture was used) was put into the tube, and 5 min after incubation, 5% TCA was added. The precipitate was collected on glass fiber filter (Whatman, GF-F), and its radioactivity was measured by a liquid scintillation counter.

T4 ghosts were provided by Dr. K. Okamoto.

Preparation of T4 DNA. T4 DNA was prepared by phenol extraction of phage T4D which had been purified by differential centrifugation. Equal volume of ethyl ether was added to the aqueous layer to remove phenol. The resulting DNA suspension was dialyzed against 2xSSC (SSC contains 0.15M NaCl and 0.015M sodium citrate). Before use for DNA-RNA hybridization-competition experiments, DNA was denatured by heating in boiling water for 10 min.

Preparation of T4 CM-RNA (RNA synthesized in the presence of chloramphenicol). For RNA preparation, we applied the procedures described by Okamoto *et al.* (1962) with a slight modification. *E. coli* B was infected with phage T4D at a m.o.i. (multiplicity of infection) of 5 in the presence of chloramphenicol (125 µg/ml). After 10 min of incubation at 30°, NaN₃ (0.01M) was added to the culture. The harvested cells were suspended in 0.01M Tris-HCl, pH7.5, 0.005M MgCl₂, 0.005M NaN₃. In the presence of DNase (20 µg/ml) and lysozyme (200 µg/ml), the suspension was frozen and thawed for 3 times, and then acetate buffer (0.03M, pH5.2) and SDS (0.4%) were added. RNA was phenol-extracted and precipitated by the addition of ethyl alcohol. The precipitate was washed twice with 75% and 99% ethyl alcohol, and dissolved in 2xSSC.

Pulse labeled T4 RNA. *E. coli* B previously UV-ray irradiated in order to inhibit host RNA synthesis were infected or superinfected with phage at a m.o.i. of 5, and pulse

labeled at different times as indicated in the legend to Fig. 5 with [^{14}C]uridine (500 mCi/mmol, 1.25 $\mu\text{Ci/ml}$ culture was used). The labeling was terminated by the addition of NaN_3 (0.01M), and labeled RNA was extracted as described above.

DNA-RNA hybridization-competition. Hybridization-competition was carried out in solution (Nygaard and Hall, 1963, 1964). Denatured DNA (5 μg), labeled RNA (1-2 μg), and CM-RNA (competitor, 0-90 μg) were incubated in a small tube (total volume, 0.3 ml of 2xSSC) for 18 hr at 68°. Then, the tube was vigorously shaken and RNA not hybridized was digested by incubation with heat-treated pancreatic RNase (40 $\mu\text{g/ml}$) for 15 min at 37°. The sample was filtered through nitrocellulose filter paper, and it was washed with about 50 ml of 2xSSC. The dried filter paper was put into a vial containing 5 ml of toluene-diphenyloxazole scintillant, and its radioactivity was measured in a liquid scintillation counter.

Gene expression of superinfecting phage.

i. imm gene. E. coli B was primarily infected with T4Dimm2 at a m.o.i. of 5, and at different time intervals, superinfected with T4D (m.o.i.=5). After 10 min of incubation at 30°, chloramphenicol (125 $\mu\text{g/ml}$) was added to the culture. The immunity of these cells was measured as described above.

ii. s gene. Phage infection was done as in the case of imm gene except that the first phage T4Dimm2-s2 and the second phage T4Dimm2 were used instead of T4Dimm2 and T4D, respec-

tively. After addition of chloramphenicol (125 $\mu\text{g}/\text{ml}$), the resistance of phage-infected cells against ghosts was tested by the same method as that for measurement of imm gene expression, since we could take advantage of the fact that T4imm2-s2-infected cells were more sensitive to ghosts than T4imm2-infected cells (Okamoto and Yutsudo, 1974).

iii. gene 30 (DNA ligase) and 33 (late gene control).

E. coli B (no amber suppressor) was infected primarily with T4Dimm2-tsL13-amH39(30) or T4Dimm2-tsL13-amN134(33) at a m.o.i. of 4, and secondarily with T4DamB22(43) at a m.o.i. of 4 at different time intervals at 30° (at this temperature, the ts mutation does not affect phage multiplication). Five minutes after second infection, an aliquot of this cell culture was plated on E. coli B, and the number of infective centers was counted.

RESULTS

SDS-Polyacrylamide Gel Electrophoresis of imm Gene Products

T4D- and T4Dimm2-induced polypeptides were analyzed by means of SDS-polyacrylamide gel electrophoresis. Polypeptides were labeled with [³⁵S]methionine during the first 10 min after phage infection. Host protein synthesis was repressed by UV-ray irradiation (data not presented). As shown in Fig. 1, at least three polypeptides which were present in T4D-infected cells were not detected in T4Dimm2-infected cells (imm-a, imm-b, and imm-c; subsequently these are called imm gene products for convenience, although it is unknown whether or not all these polypeptides participate in the establishment of immunity). In contrast, one other polypeptide, imm-d, which was not induced by wild-type phage appeared when cells were infected with T4Dimm2. Judging from their molecular weights, this polypeptide was thought to be a short fragment derived from imm-a. A similar electrophoretic pattern was also obtained when [¹⁴C]amino-acid mixture was used instead of [³⁵S]methionine.

Molecular weights of these polypeptides imm-a, imm-b, imm-c, and imm-d were estimated to be 77,000, 45,000, 33,000, and 72,000, respectively (Fig. 1'), in comparison to the mobility, on SDS-gel, of polypeptides of Adenovirus type 2 virions (Ishibashi and Maizel, 1974).

Time Course of Appearance of imm Gene Products

T4D-infected cells were pulse labeled with [³⁵S]methionine at different times after infection and the samples were subjected to SDS-polyacrylamide gel electrophoresis. The results were shown in Fig. 2. The kinetics of synthesis of three imm gene products were determined by densitometric scanning of this autoradiogram (Fig. 2'). This showed that the synthetic rate of each imm gene product increased linearly up to about 10 min after infection, and thereafter it stayed constant.

It has been reported that the exclusion is brought about by phage-directed protein which is a product of immediate-early mRNA (Yutsudo and Okamoto, 1973), and that it is almost completely established at a very early stage of infection (within about 3 min after infection at 30°) as measured by superinfection breakdown (Graham, 1953), complementation test (Dulbecco, 1952), or resistance against phage ghosts (Vallée *et al.*, 1972). However, one could hardly detect the imm gene products on SDS-gels until a few minutes after infection. The explanation of this discrepancy is that these imm gene products were not synthesized in a detectable amount on SDS-gels at an early stage of infection, since none of the other early polypeptides were detected either under these conditions (Fig. 2).

Shutoff of imm Gene Expression of Superinfecting Phage

In order to study the regulatory mechanism of imm gene expression, we examined the immunity of the cells that had been infected primarily with T4Dimm2 and, after a while, secondarily with T4D (imm^+). The results are shown in Fig. 3. When cells were superinfected with T4D within 2 min after primary infection, immunity was established, while, when imm gene was introduced into T4Dimm2-infected cells by superinfection with T4D at about 3 min or later after primary infection, it was no longer expressed.

This shutoff is not due to the unavailability of superinfecting phage DNA, since Daegelen and Brody (1976) have shown the synthesis of delayed-early (rIIA) and quasi-late (rIIB) mRNA of superinfecting phage.

Shutoff of imm Gene Products As Analyzed by SDS-polyacrylamide Gel Electrophoresis

We analyzed first or second phage-induced polypeptides by means of SDS-polyacrylamide gel electrophoresis (Fig. 4). As described above, T4Dimm2-infected cells lacked three polypeptides (imm-a, imm-b, and imm-c) but contained one other polypeptide (imm-d) which was not detected in T4D-infected cells (Fig. 4a and 4b). When T4Dimm2 and T4D were added simultaneously to cells, these four polypeptides were synthesized (Fig. 4e), whereas, if superinfection with T4D was performed at several minutes after primary infection, no imm gene

products, except for imm-d, were detected (Fig. 4d). A polypeptide that had a similar mobility to imm-b on SDS-gels appeared at a late stage of infection (Fig. 4c and 4d). However, it may be one of the late proteins which is independent on imm gene, since T4Dimm2 did also produce it (Fig. 4c).

These results eliminate the possibility that a failure in the establishment of immunity by superinfecting phage is due to competition between normal and defective immunity protein(s) for acting sites, because the normal products are not synthesized under this condition. Fig. 4 also shows that the second phage does not affect protein synthesis of the first phage, since the electrophoretic pattern of the polypeptides synthesized at later period (between 10 and 20 min after primary infection) was not altered by superinfection (Fig. 4c and 4d).

A Phage-directed Factor(s) Controlling the Shutoff

As the shutoff was expected to be brought about by a primary phage-induced protein(s), we undertook the following experiments.

First, we studied the effects of UV-ray irradiation of cells or phage on this shutoff. As shown in Table 1a, when UV-ray irradiated cells were infected with the intact phage, the shutoff occurred normally. This suggests no requirement of host protein synthesis for the shutoff. On the other hand, UV-ray irradiated phage damaged considerably the cells.

Further, we examined the shutoff in the presence of chloramphenicol. After the first and the second phage infections, chloramphenicol was removed from a medium by means of filtration. The cells were suspended in a medium containing no chloramphenicol, and incubated, followed by ghost infection to measure the immunity. As seen in Table 1b, the shutoff was considerably, not completely, blocked by chloramphenicol. This incompleteness could be explained by the fact that phage infection in the presence of chloramphenicol, as well as UV-ray irradiated phage infection, damages the cells (Saijo and Okamoto, 1976). Nevertheless, from the results in Table 1, it is likely that a phage-directed protein plays a role in this shutoff.

DNA-RNA Hybridization-competition

The shutoff of imm gene expression of superinfecting phage began within 2 min after infection as described above. On the other hand, early transcriptional changes occur almost at the same time as this shutoff (Grasso and Buchanan, 1969; Salser et al., 1970), and imm gene belongs to the immediate-early class (Yutsudo and Okamoto, 1973). Therefore, studies on this shutoff may provide some valid suggestion to interpret the switching mechanism from immediate-early to delayed-early transcription.

First, it was examined whether this shutoff is specific to imm gene or it applies to all immediate-early genes.

In the case of a single infection, it is well known that a considerable proportion of RNA which is labeled at a later stage of infection is competed out by immediate-early mRNA in DNA-RNA hybridization experiments (Salser et al., 1970; Young, 1975). We also confirmed this (Fig. 5). This suggests that, in this case, some immediate-early mRNA is synthesized even after the switching on of delayed-early transcription.

After the shutoff of imm gene expression is induced by the first phage, are any immediate-early genes transcribed? To test this, RNA which was labeled after superinfection was analyzed by DNA-RNA hybridization-competition. If immediate-early genes of superinfecting phage are shut off, the proportion of immediate-early mRNA in total mRNA will not change whether cells are superinfected or not. The results were shown in Fig. 5. When cells were superinfected 10 min after primary infection and labeled with [¹⁴C]uridine for the first 2 min after superinfection, the proportion of immediate-early mRNA augmented as compared with that prepared from the cells which were not superinfected. This implies the possibility that some immediate-early genes of superinfecting phage were transcribed even after the shutoff of imm gene expression occurs.

Expression of Other Genes of Superinfecting Phage

The expression of three early genes (s, 30, and 33) of superinfecting phage was indicated by using phage mutants,

T4Dimm2-s2, T4Dimm2-tsL13-amH39(gene 30), and T4Dimm2-tsL13-amN134(gene 33). Each gene expression was tested as described in MATERIALS AND METHODS. As shown in Fig. 6 and Fig. 7, the shutoff of these three genes of superinfecting phage did not occur even if the time interval between primary and secondary infections was extended up to 8 min. From these results in addition to those of hybridization-competition experiments, it is apparent that the shutoff of imm gene expression is not due to the inactivation of superinfecting phage DNA.

Although gene 33 (late gene control) has not been classified, it seems to be a delayed-early gene on the basis of its function, while genes s [suppression of lysozyme gene (Emrich, 1968)] and 30 (DNA ligase) belong to the immediate-early class (Peterson et al., 1972; Jayaraman, 1972). Therefore, there would be at least two different immediate-early classes with respect to regulatory mechanism of gene expression; one includes imm gene, the other s and 30.

DISCUSSION

Three Polypeptides Missing in T4Dimm2-infected Cells

By SDS-polyacrylamide gel electrophoresis, at least three polypeptides, imm-a, imm-b, and imm-c having molecular weights of 77,000, 45,000, and 33,000, respectively, were shown to be missing in T4Dimm2-infected cells in comparison with T4D-infected cells (Fig. 1 and Fig. 1'). One other polypeptide, imm-d, whose molecular weight was 72,000 was induced only by T4Dimm2, and could be considered to be a short fragment of imm-a.

O'Farrell and Gold (1973) have also reported two immunity proteins having molecular weights of 40,000 and 28,000. These two proteins would be the same as imm-b and imm-c judging from their electrophoretic patterns and from the fact that the same phage T4imm2 was used to identify imm gene products in comparison with T4D-induced proteins on SDS-gels.

Since the type of mutation of T4Dimm2 is unknown, the relationship among three polypeptides, imm-a, imm-b, and imm-c, is not fully established. However, it should be noteworthy that kinetic studies showed the similar pattern of synthesis of these three polypeptides (Fig. 2 and Fig. 2'). Further, when cells were superinfected with T4D (imm+) 10 min after primary infection with T4Dimm2, all of these three polypeptides were not synthesized (Fig. 4).

From these results, we feel like considering that they have a close correlation each other.

Imm gene is shown to situate between genes 42 and 43 on the linkage map of phage T4 (Cornett and Vallée, 1973; Childs, 1973), and no other genes have been mapped in this region. One could imagine that the polypeptides identified in the present study might include gene 42 or 43 products. However, this is unlikely because (i) a mutation imm2 does not affect the function of genes 42 and 43 (immunity mutation is not lethal), and (ii) products of genes 42 and 43 are completely different from those of imm gene on SDS-gels (data not presented). Until the present, two immunity mutants, T4imm1 (Childs, 1973) and T4imm2 (Vallée and Cornett, 1972), have been isolated, and their mutations have been shown to locate in the same allele on the basis of complementation experiments (Cornett and Vallée, 1973). However, this does not always mean that there is only one gene responsible for immunity.

With respect to the question whether all three polypeptides described above participate in the establishment of immunity, we have no other information at present. It requires further studies for answering this question.

Shutoff of imm Gene Expression of Superinfecting Phage

In a few minutes after primary infection, the infected cells became to lose the ability to make express imm gene of superinfecting phage. This shutoff of imm gene expression

of superinfecting phage was shown through both measurement of immunity and polypeptide analysis (Fig. 3 and Fig. 4). We showed that it required no host protein synthesis but phage-directed one for the shutoff of imm gene expression (Table 1), of which role, though, is not clear. Whether or not imm gene expression of the first phage is also under the same control as in the case of superinfecting phage is an unsolved problem. The expression of other early genes such as genes s, 30, and 33 was not affected by primary infection (Fig. 6 and Fig. 7).

Although it is difficult to state unambiguously the mechanism of this shutoff, it is most likely that transcription of imm gene is shut off a few minutes after primary infection on the basis of the following points of view. (i) This shutoff is not a result of an inactivation or abnormality of transcriptional or translational machinery caused by superinfection, since Daegelen and Brody (1976) have reported that mRNA coded for D, rIIA, and rIIB of superinfecting phage are synthesized immediately after introduction of their genes into preinfected cells, and since some other early genes of superinfecting phage were shown to be expressed (Fig. 5, Fig. 6, and Fig. 7). (ii) Nor is the shutoff interpretable by competitive inhibition of normal imm gene products by defective one from the first phage T4Dimm2, because electrophoretic studies showed the shutoff of the synthesis of normal products

themselves after infection (Fig. 4). (iii) In the case of a single infection with T4D, synthesis of imm gene products increased in its amount up to at least 10 min after infection as shown in Fig. 2', suggesting that if normal imm gene mRNA exists in the infected cells, the protein synthesis would not be shut off, although we can not eliminate the possibility that imm gene messenger of superinfecting phage is different from that in the case of a single infection.

However, in order to prove the shutoff of imm gene expression at the transcriptional level, it requires more direct evidences such as analysis of mRNA species synthesized after superinfection or of polypeptides produced in a cell-free system. In spite of our trials along this line, we have not obtained a clear-cut result thus far.

Postulates for the Regulation of imm Gene Expression

It is apparent that the elongation theory does not fit to account for this shutoff, because, according to this theory, immediate-early transcription is not arrested at an early stage of infection.

It is, however, possible to interpret this phenomenon by the new promotor theory, that is, after some changes in transcriptional events occur, RNA polymerase can not recognize promoters for immediate-early genes but can do promoters for delayed-early or quasi-late genes. Travers (1969) has reported that T4-coded sigma factor represses immediate-early

transcription and stimulates delayed-early mRNA synthesis. However, the fact that this factor is not detected within a few minutes after infection at 30°, but appears between 5 min and 15 min (Travers, 1969), rules out the participation of this factor in the regulation of imm gene expression. A diffusible product has recently been reported to control delayed-early and quasi-late transcriptions (Daegelen and Brody, 1976), but the mode of its action has not yet been clearly resolved.

As there were at least two different immediate-early classes with respect to the regulatory mechanism of their expression, it is difficult to suppose a modified RNA polymerase in order to explain these two different mechanisms. Therefore, regarding the shutoff of imm gene expression, we are rather inclined to consider a negative factor which blocks promoter for imm gene or modifies it so as to change its affinity to RNA polymerase. In any case, it seems to be almost impossible to resolve the switching from immediate-early to delayed-early transcription by only one mechanism.

It is also possible to think that defective product(s) of imm gene of primary phage T4Dimm2 inhibits transcription or translation of imm gene. If that is the case, one would expect that the defective products themselves will not be synthesized in a large quantity, contrary to the fact that one of these products, imm-d, was synthesized as much as imm-

a (Fig. 1).

Another possible interpretation on this shutoff will be derived from the fact that DNA replication origin situates in the genetic region between genes 40 and 43, and that these genes are attached preferentially to cell membrane (Marsh et al., 1971). It is known that imm gene locates between genes 42 and 43 on the genetic map of phage T4 (Cornett and Vallée, 1973; Childs, 1973), namely, it is just adjacent to the replication origin. If imm gene of primarily or secondarily infecting phage is attached to membrane a few minutes after infection, and if it is no more transcribed after its attachment to membrane, the shutoff of imm gene expression could be explained. In our preliminary experiments, the expression of gene 42 of superinfecting phage was also shown to be shut off 5 min after primary infection by means of a complementation test (data not presented). This observation implies a possibility that DNA replication has a relation to these shutoff.

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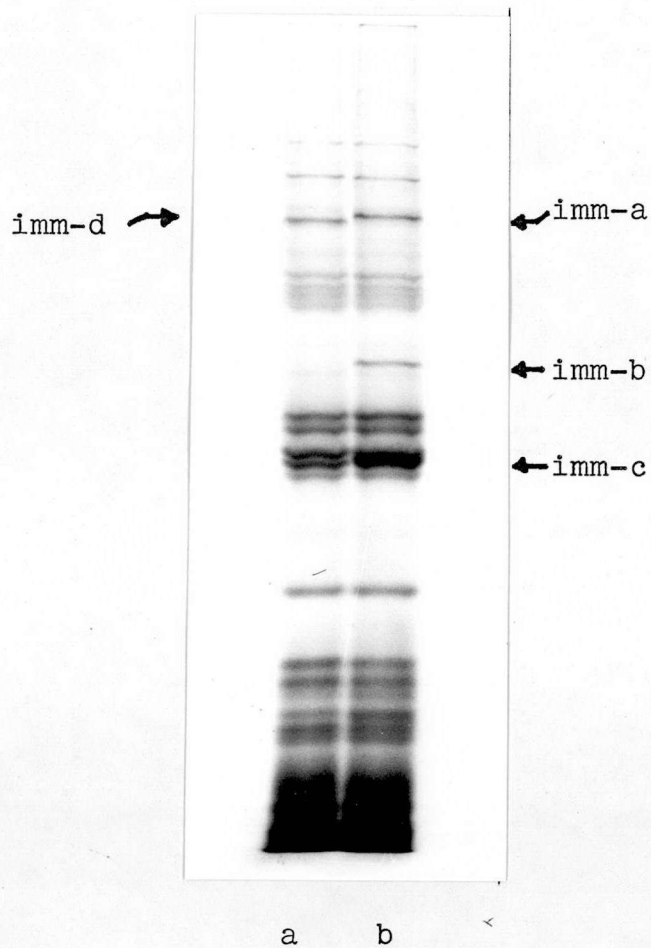


Fig. 1. Autoradiogram of T4D- and T4Dimm2-induced polypeptides analyzed by SDS-polyacrylamide gel electrophoresis. Phage-infected cells were labeled with [^{35}S]-methionine (406 Ci/mmol, 20 $\mu\text{Ci/ml}$ culture was used) for first 10 min after infection at 30°. a: T4Dimm2-induced polypeptides. b: T4D-induced ones. (See MATERIALS AND METHODS)

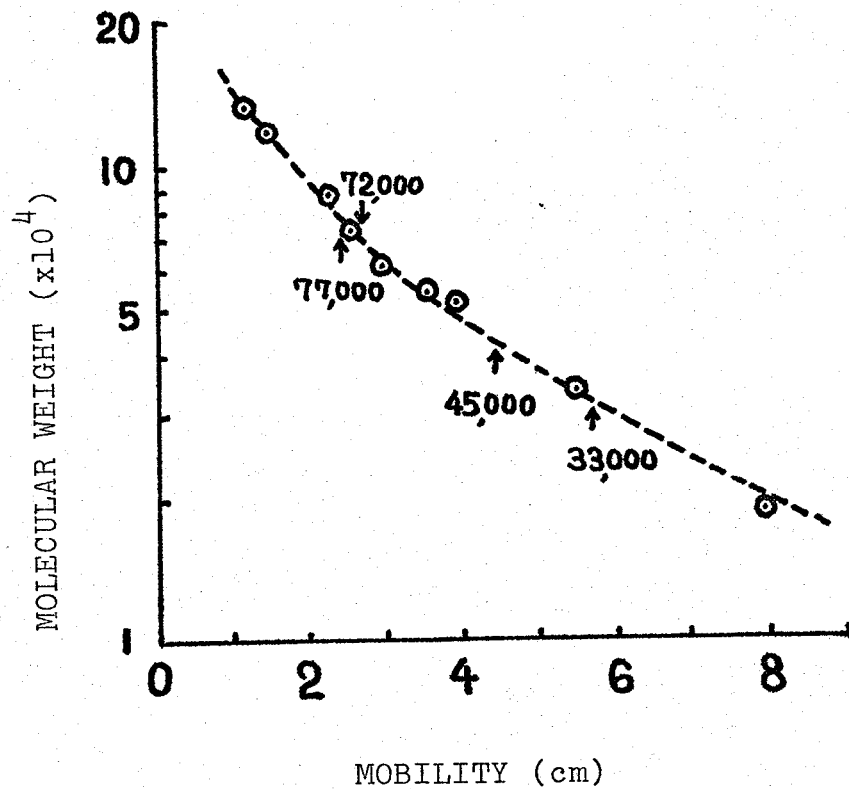


Fig. 1'. Molecular weights of imm gene products estimated by their mobilities on SDS-polyacrylamide gel (10%). For standard markers, Adenovirus type 2 virions were used. Imm-a, 77,000; imm-b, 45,000; imm-c, 33,000; imm-d, 72,000.

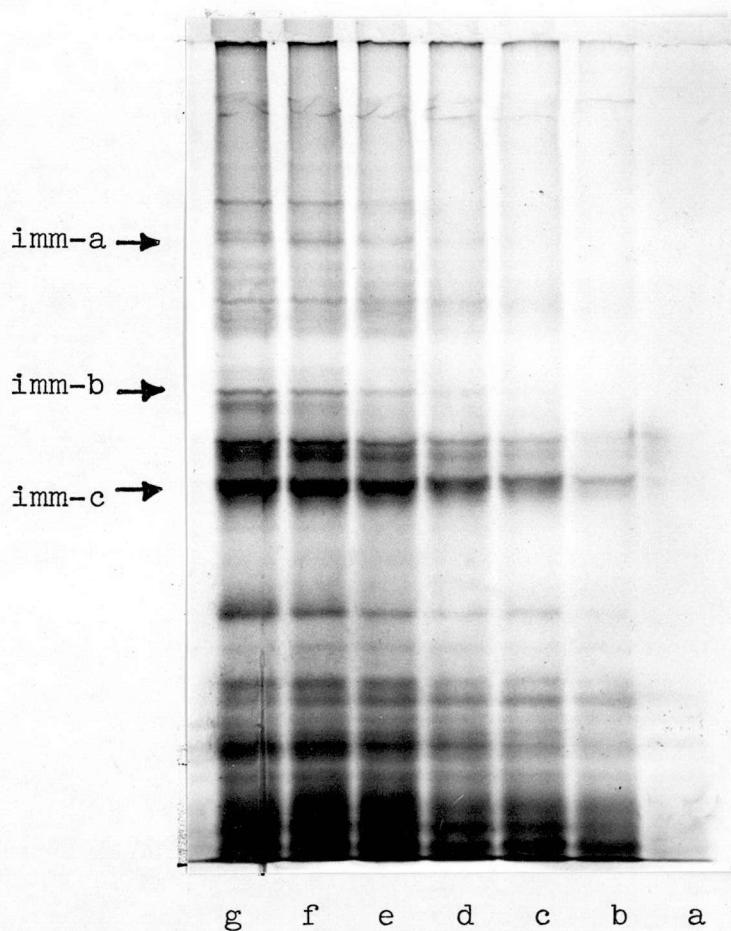


Fig. 2. Time course of synthesis of imm gene products. Cells were infected with T4D and labeled with [^{35}S]-methionine (406 Ci/mmol, 30 $\mu\text{Ci/ml}$ culture was used) during appropriate periods after phage infection as indicated below at 30°. a: 0-2 min. b: 2-4 min. c: 4-6 min. d: 6-8 min. e: 8-10 min. f: 10-15 min. g: 15-20 min. Labeled samples were subjected to SDS-polyacrylamide gel electrophoresis as described in MATERIALS AND METHODS.

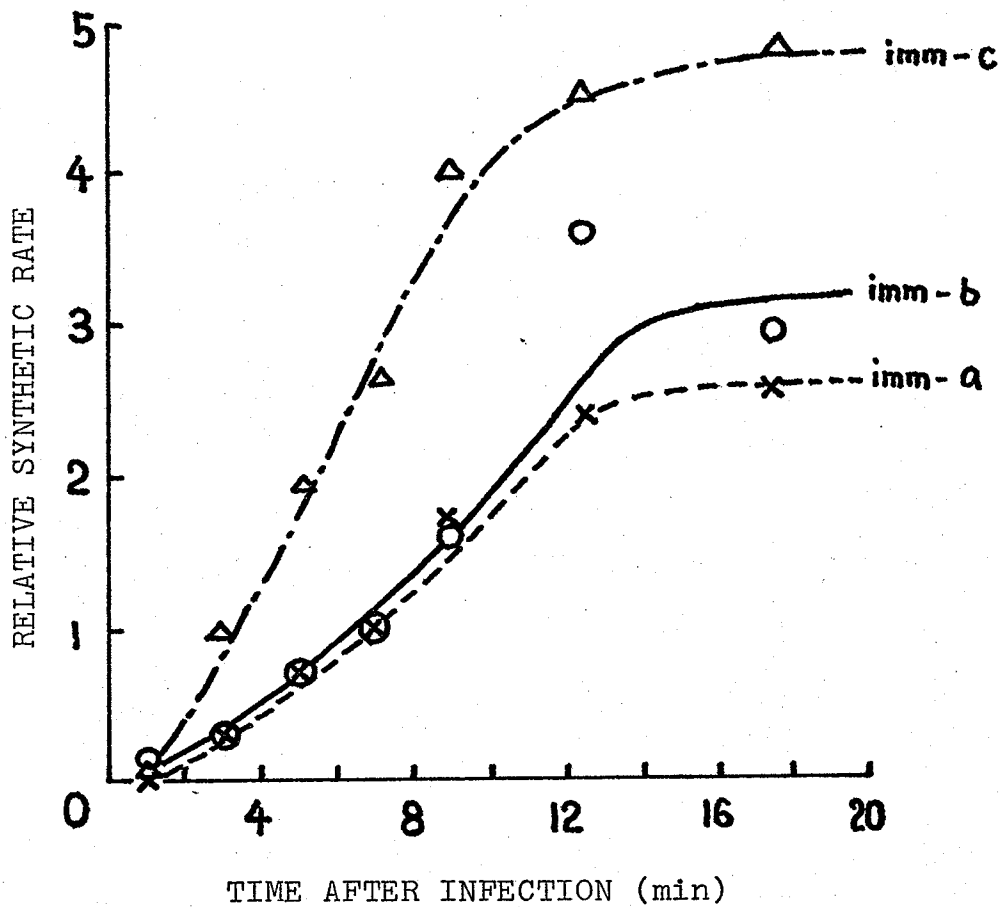


Fig. 2'. Time course of synthesis of imm gene products (densitometric data). The kinetics was determined by densitometric scanning of the autoradiogram in Fig. 2. Regarding imm-c, ordinate does not show the value itself, because the band of imm-c and other adjacent bands overlapped one another on SDS-gel.

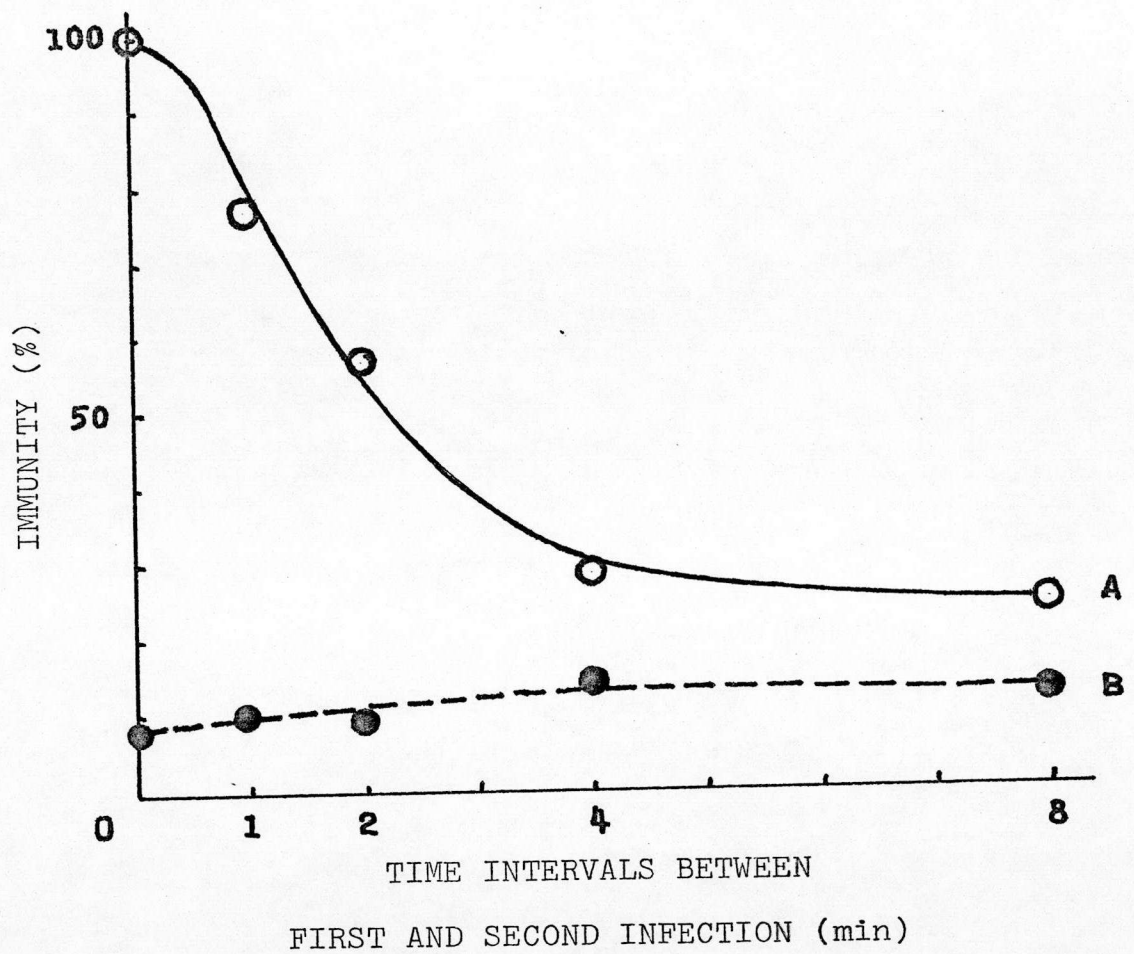


Fig. 3. Shutoff of *imm* gene expression of super-infecting phage. A: primary phage, T4Dimm2 (m.o.i.=5); secondary phage, T4D (m.o.i.=5). B: primary phage, T4Dimm2 (m.o.i.=5); without superinfection. The immunity of infected-cells was measured as described in MATERIALS AND METHODS.

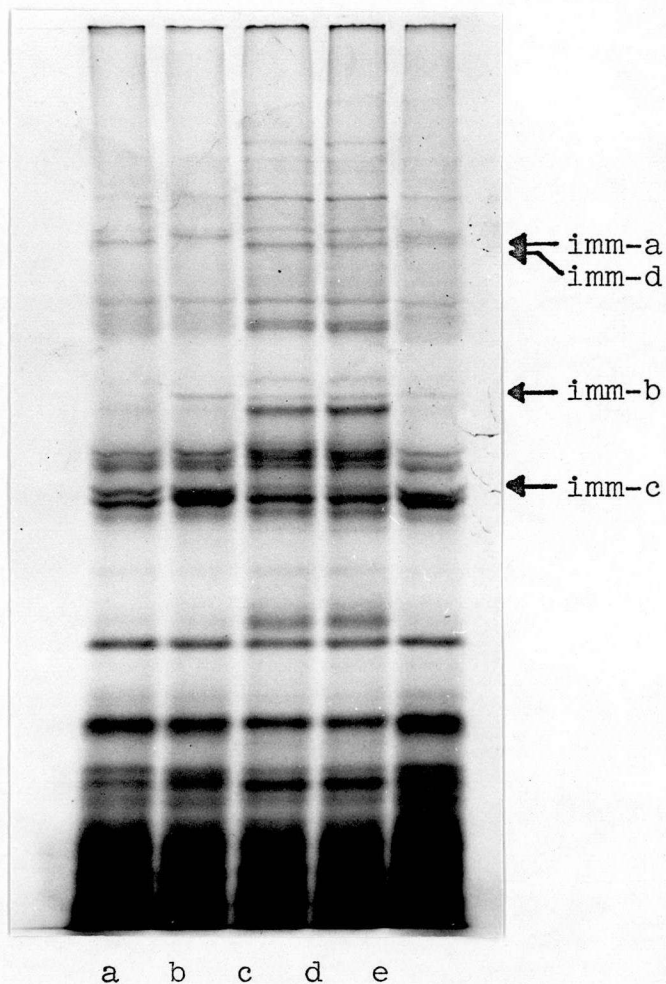


Fig. 4. Shutoff of imm gene products of superinfecting phage. Phages and labeling periods were indicated below. Infected cells were labeled with [^{35}S]-methionine (406 Ci/mmol, 20 $\mu\text{Ci/ml}$ culture was used) at 30°. a: infected with T4Dimm2, labeled 0-10 min after infection. b: with T4D, 0-10 min. c: with T4Dimm2, 10-20 min. d: primarily with T4Dimm2 and secondarily with T4D at 10 min interval, 10-20 min after primary infection. e: with T4Dimm2 and T4D, 0-10 min. Phage infection was done at a multiplicity of 6. For experimental details, reference to MATERIALS AND METHODS.

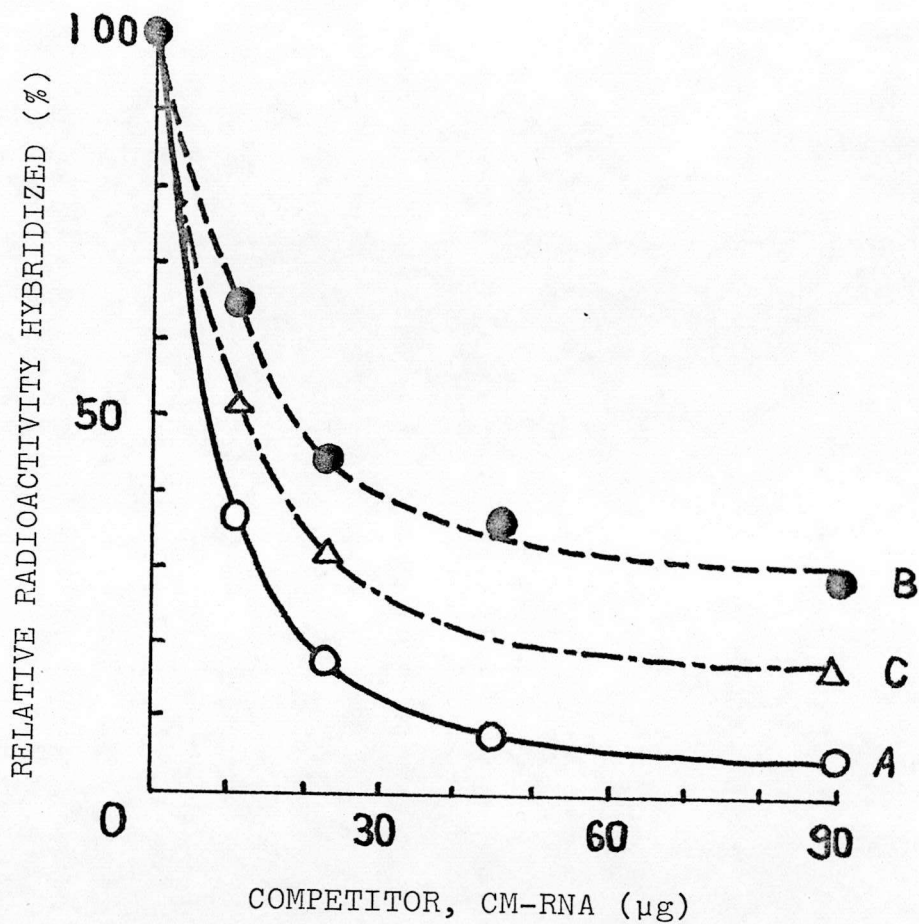


Fig. 5. DNA-RNA hybridization-competition experiments. Labeled RNA was prepared from cells (A) infected with T4DamN134, and labeled 0-2 min after infection, (B) infected with T4Dimm2-tsL13-amN134, and labeled 10-12 min after infection, (C) infected primarily with T4Dimm2-tsL13-amN134, and at 10 min interval secondarily with T4DamN134, and labeled 10-12 min after primary infection. The labeled mRNA would not contain late mRNA, since gene 33 controls late gene expression. Other experimental details were described in MATERIALS AND METHODS.

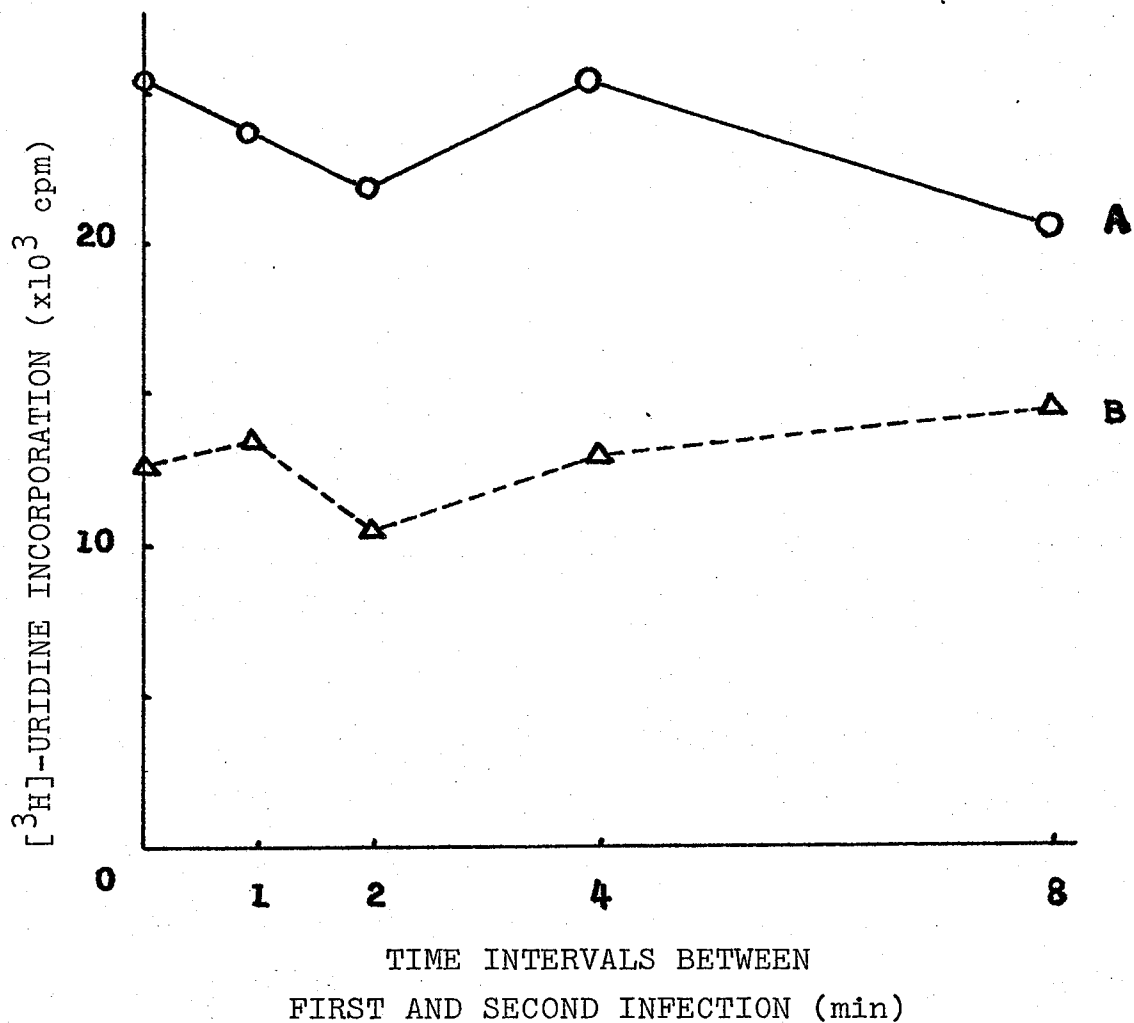


Fig. 6. Expression of s gene of superinfecting phage. First and second phages used were indicated below. A: first, T4Dimm2-s2 (m.o.i.=5); second, T4Dimm2 (m.o.i.=5). B: first, T4Dimm2-s2 (m.o.i.=5), second, T4Dimm2-s2 (m.o.i.=5). Degree of s gene expression can be given by amount of [³H]-uridine incorporated into cells superinfected with ghosts (see MATERIALS AND METHODS).

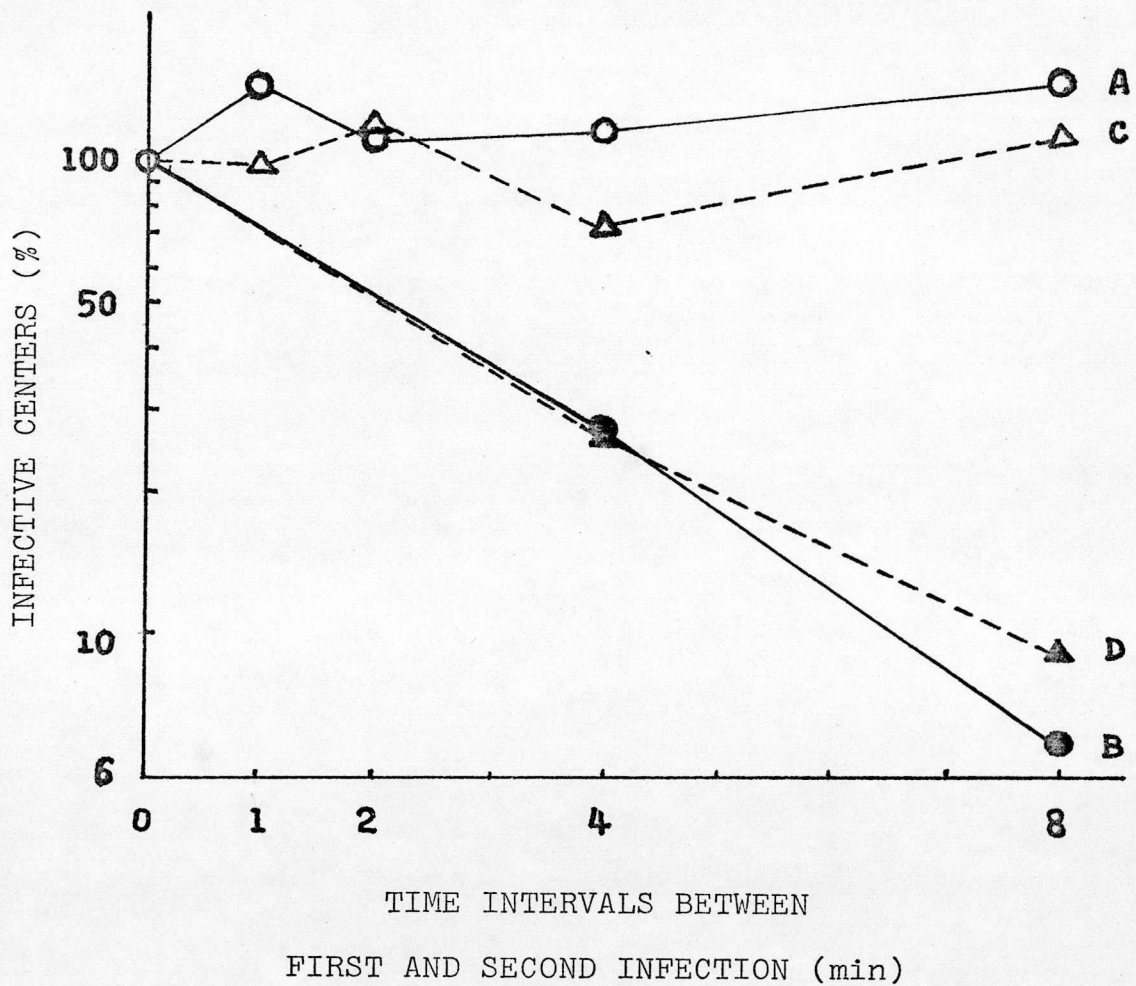


Fig. 7. Expression of genes 30 and 33 of superinfecting phage. First phages used were listed below. For second phage, T4DamB22 was used at a multiplicity of 4. A: T4Dimm2-tsL13-amH39(30) (m.o.i.=4). B: T4DamH39 (m.o.i.=4). C: T4Dimm2-tsL13-amN134(33) (m.o.i.=4). D: T4DamN134 (m.o.i.=4). (See MATERIALS AND METHODS).

TABLE 1

A Phage-directed Protein(s) Controls the Shutoff of imm Gene Expression

a				b			
	first	second	immunity		first	second	immunity
UV-ray irradiated cells	T4D	T4D	94.4 (%)	-CM	T4D	T4D	100.4 (%)
	T4Dimm2	T4Dimm2	43.5		T4Dimm2	T4Dimm2	26.7
	T4Dimm2	T4D	58.3		T4Dimm2	T4D	32.4
UV-ray irradiated first phage	T4D	T4D	57.7	+CM (125 µg/ml)	T4D	T4D	28.4
	T4Dimm2	T4Dimm2	42.5		T4Dimm2	T4Dimm2	2.3
	T4Dimm2	T4D	45.2		T4Dimm2	T4D	13.8

Legend to TABLE 1.

E. coli B was infected with phage indicated in the table at a multiplicity of 6 at 30°. Cells were superinfected 8 min after primary infection, and incubated for further 10 min after second infection (b) or after suspension in a fresh medium (b). In the case of (b), chloramphenicol (CM) was removed by means of filtration 3 min after second infection. The immunity of these cells was measured as described in MATERIALS AND METHODS.