ROLES OF GENE 45 PRODUCT INTO T4 DNA REPLICATION AND LATE GENE EXPRESSION OF: TEMPERATURE REVERSIBILITY EFFECT

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1. Introduction

The expression of the late genes during bacteriophage T4 development requires concomitant phage DNA replication [1] as well as the function of the products of the genes 33 and 55 [2,3]. These two phage proteins were also shown to copurify with the host RNA-polymerase [4–6].

Substantial late gene expression, however, can be achieved in vivo even in the absence of concomitant phage DNA replication by introducing into the infecting phage mutations in the T4 DNA-polymerase (gene 43), T4 DNA-ligase (gene 30) and T4 exonuclease (genes 46 or 47) [7,8].

Under those conditions late messenger RNA (mRNA) synthesis is uncoupled from DNA replication and it was found that the function of another early T4 gene product (gene 45) beside the function of the gene 33 and 55 products is continuously required to allow late gene expression [9,10].

Also the existence was proved of a specific binding of the gene 45 protein to agarose coupled host RNA-polymerase extracted from T4 infected cells [11].

The gene 45 product, however, has a second role in T4 development: in vivo, it is absolutely and continuously required for the T4 DNA replication [1,12]; in vitro, Alberts and coworkers [13] achieved extensive replication of T4 DNA by a complex of the purified T4 replication proteins including the gene 45 product.

In this paper we present data on the temperature reversibility effect of the temperature sensitive product of gene 45 (tsp45) on DNA synthesis and late gene expression under conditions where late transcription is coupled or uncoupled to DNA replication. We conclude that upon temperature shift-down the tsp45 synthesized at 42.6°C is functional only for DNA replication, while it does not allow late transcription to occur.

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2. Materials and methods

E. coli B^E cells grown at 37°C in M9 [14] at a concentration of 4×10^8 cells/ml were spun down, washed and resuspended in M9 buffer at a concentration of 1.5×10^9 cells/ml. Cells were then infected at 37°C with the temperature sensitive mutant at an average m.o.i. of 8. Seven minutes later adsorbtion was complete: 1% of the input phages were still able to form plaques under permissive conditions and the surviving bacteria were less than 1%. The infected cells were then diluted 5-fold in complete M9 medium prewarmed at 30°C or 42.6°C, respectively: this was assumed as infection starting time. At various times temperature shifts of portions of the culture were performed by a 4-fold dilution in complete M9 medium prewarmed at the appropriate temperature.

The phages used and their phenotypes are listed in table 1.

The temperature sensitive gene 45 mutants used in this work had been backcrossed three times against T4 wild type and proved to contain independent mutations by measuring the frequency of ts^+ phages produced by recombination experiments.

DNA synthesis, RNA preparation and RNA/DNA hybridization experiments were respectively performed

Table 1
Properties of phage T4 mutants

Mutant	Gene	Phenotype	Function
tsCB53, tsP73CT, tsA89, tsA76	45	DO	Component of DNA replication complex also associated with host RNA-polymerase
tsP36, am4301	43	DO	DNA-polymerase
amH39X	30	DA	Polynucleotide ligase
amN130	46	DA	Exonucleolytic function

Abbreviations: DO, no replication; DA, replication arrested; MD, maturation (i.e. late functions) defective

following standard procedures [1,9,14]. The analysis of intracellular T4 DNA was performed according [15].

3. Results

3.1. *Reversibility of temperature-sensitive gene 45 mutants*

E. coli B^E cells were infected at the non-permissive temperature with a number of different temperature-sensitive gene 45 mutants. At various times after infection a portion of the infected cells was brought to 30°C and viable phage production was measured 1 h later.

Table 2 shows the results of this experiment along with a tsP36 (gene 43) mutant as control. The data indicate a drastic effect on the burst size for all the ts45 mutants with respect to the ts43 mutant.

3.2. *DNA synthesis*

The first question we want to answer is if DNA replication resumes after lowering the temperature and if the tsp45 made at 42.6°C can be reversibly reactivated.

For the next set of experiments the data were obtained by using the tsCB53 mutant: analogous results were obtained with the other temperature sensitive mutants listed in table 1.

Figure 1 shows the DNA synthesis measured through the accumulation of the [^{14}C]TdR into the alkali-insoluble linkages of *E. coli* B^E cells infected with the tsCB53 and with the tsP36 mutants, with and without chloramphenicol (CAM) addition, following the experimental scheme illustrated in the

Table 2
Phage yields in tsp45 and tsp43 infections

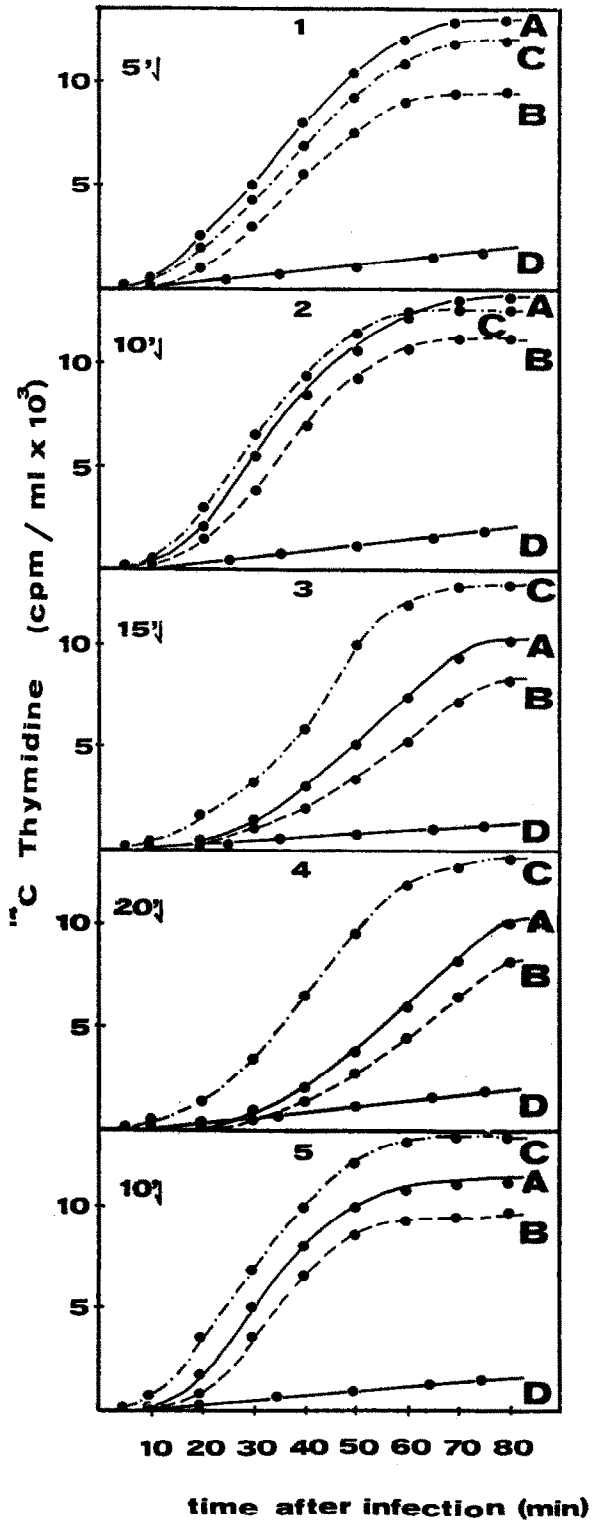
Part 1

Time of shift-down	tsCB53 A	tsP73 B	tsA89 C	tsA76 D	tsP36 E
2′	80	90	117	76	130
4′	60	60	88	72	–
6′	50	40	75	57	120
8′	35	5	24	40	–
9′	23	0.5	9	12	–
10′	10	0.5	4	6	110
12′	4	0.9	0.4	4	–
15′	2	0.4	0.1	1	90
20′	0.4	0.3	0.1	0.2	80

Part 2

Temperature of infection (°C)					
30	120	125	130	100	140
42.6	0.3	0.1	0.4	0.1	0.1

E. coli B^E cells grown as described in Materials and Methods were infected at 42.6°C with the appropriate phage mutants. At various times after infection, portions of the infected cells were shifted down to 30°C. Phage yields were measured 60 min after shift-down by addition of $CHCl_3$ and plating on *E. coli* B^E at 30°C. Part 1: Columns A, B, C and D show the phage yields of the different mutants used in gene 45; column E shows the phage yield of the mutant in the DNA-polymerase (gene 43). The times of shift-down are indicated in the first column. Part 2: The phage yields of the same mutants under permissive (30°C) and non-permissive (42.6°C) conditions are shown in line 1 and line 2, respectively



previous section. Upon temperature shift-down the DNA synthesis starts immediately at a normal rate if the shift-down is done not later than 10 min after infection (panel 1 and 2). In all cases (panel 3 and 4) it reaches the level of the DNA synthesis of the portion of the infected culture left at the permissive temperature.

Addition of CAM 1 min before the shift-down to prevent further protein synthesis also allows substantial DNA replication to occur. Panel 5 shows as a control the analogous experiment done with the tsP36 mutant. In all the following experiments the shift-down was done 10 min after infection.

Since it has been shown that T4 DNA replicates at least partially as a linear DNA concatemer [15–17] we have further analyzed the intracellular DNA synthesized in ts45 infection.

Figure 2 shows that the DNA synthesized after shift-down (panel A) contains single strand DNA molecules with a higher molecular weight than native T4 DNA strands. When CAM is added 1 min before the shift-down, high molecular weight DNA accumulates as well (panel B).

3.3. *Late gene expression*

Since the gene 45 product is also a regulatory protein required for late transcription we have first analysed the synthesis of RNA under conditions in which the late gene expression requires concomitant phage DNA replication.

Table 3 shows that, although the percentage of late mRNA synthesized after temperature shift-down is normal when protein synthesis takes place, late mRNA transcription is drastically reduced, when, by adding CAM 1 min before shift-down, further protein synthesis is prevented.

Fig.1. DNA synthesis after temperature shift-down with tsCB53 and tsP36 infected cells: chloramphenicol effect. *E. coli* B^E cells were infected at 42.6°C with tsCB53 (panels 1, 2, 3 and 4) and tsP36 (panel 5) respectively. Two minutes after infection [^{14}C]TdR (0.07 μCi/ml; 15 μg/ml) was added and the incorporation was measured as described in [1]. At the times indicated in each panel part of the infected culture was shifted down at 30°C in the absence (curves A) or presence (curve B) of chloramphenicol at a concentration of 200 μg/ml. Curves C and D represent the incorporation of cultures always kept at 30°C and 42.6°C, respectively.

The control experiment (lines 1 and 2) shows that proteins required for late transcription have accumulated at 30°C in cells infected with a ts45 mutant in sufficient amounts to substain the maximum relative rate of late messenger RNA by 10 min after infection as was already demonstrated for the T4 wild type phage [18].

In order to measure the absolute amount of late mRNA species synthesized in the presence of protein synthesis, 20′ RNA was tested by a mixed-competitor experiment.

Figure 3 shows that the RNA synthesized after temperature shift-down is a much better competitor for late mRNA species than the RNA synthesized at non-permissive temperature (compare curve C with curve B) but specific late mRNA species are either missing or much less abundant than RNA made under permissive conditions (curve D).

In order to distinguish between those two possibilities we have measured specific late proteins made after shift-down by autoradiography of [^{14}C]amino acid-labeled lysate run into SDS–polyacrylamide gel.

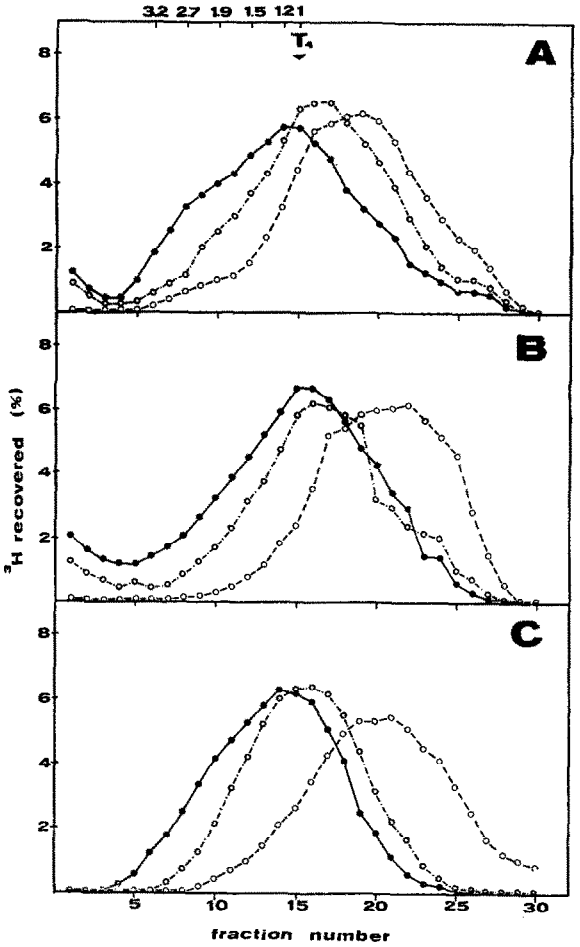


Fig.2. Sedimentation analysis of ^{3}H-labeled newly synthesized DNA in tsCB53 infected cells. *E. coli* B^E cells were infected with the tsCB53 mutant. Two minutes after infection [^{3}H]-TdR (100 μCi/ml) was added. The DNA was extracted according to [15] and analyzed in 5% to 20% alkaline sucrose gradient (SW50.1 rotor, 35 000 rev./min, 80 min, 15°C). Panel A: infection at 42.6°C, temperature shift-down was 10 min after infection. The intracellular DNA was analyzed 5 (o-o), 15 (o- · -o) and 30 (•-•) min after shift-down. Panel B: as in panel A except that chloramphenicol was added 1 min before temperature shift-down. Panel C: infection at 30°C as control; the intracellular DNA was analyzed 10 (o-o), 15 (o···o) and 20 (•-•) min after infection. Sedimentation distances are shown in the upper scale assuming as unit length intact single strand T4 DNA [21].

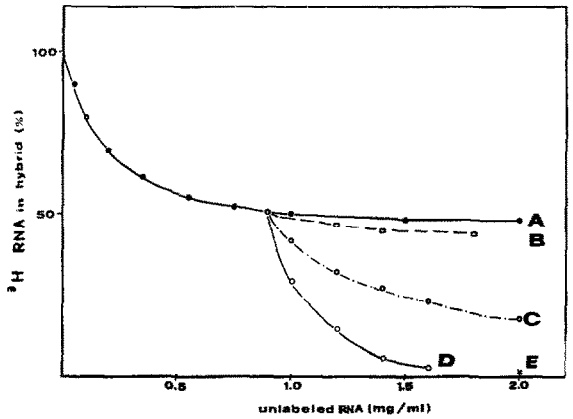


Fig.3. Mixed competition test for late messenger content of RNA extracted from *E. coli* cells infected with the tsCB53 mutant. Hybridization: all samples contained 1 μg/ml of denaturated T4 DNA, 8 μg/ml of ^{3}H-labeled late RNA (30°C, 18–22 min after infection) from wild type infected cells which were used as a probe for hybridization competition. Increasing amount of 5 min unlabeled wild type RNA competitor (curve A). A constant amount of unlabeled 5 min wild type RNA (0.9 mg/ml) was added to: (1) increasing amount of RNA extracted from tsCB53 infected cells 20 min after infection at 42.6°C (curve B); (2) increasing amounts of RNA extracted from tsCB53 infected cells 20 min after infection at 30°C (curve D); (3) increasing amounts of RNA extracted from tsCB53 infected cells 30 min after infection (the culture was shifted from 42.6°C to 30°C 10 min after infection) (curve C). Point E(x) 1 mg/ml of unlabeled 5 min and 1 mg/ml of unlabeled 20 min wild type RNA.

Table 3
Temperature reversibility of late mRNA synthesis

Mutant	Time of shift-down (min)	Time of labeling (min)	Temperature of labeling (°C)	Chloramphenicol addition (200 γ/ml)	r-Transcripts (%)
tsCB53	–	50–54	30	–	50
	–	50–54	30	+	40
	None	5–7	42.6	–	3
	10	12–14	30	+	3
	10	20–22	30	+	3
	10	20–22	30	–	38
	10	28–30	30	+	5
	10	28–30	30	–	47
tsP36	None	5–7	42.6	–	5
	10	18–20	30	–	47
	10	20–22	30	+	35
	10	33–35	30	–	50
	10	38–40	30	+	35
am4301-amH39X	–	48–50	30	–	40
amN130-tsCB53	None	28–30	42.6	–	5
	10	38–40	30	+	2
	10	38–40	30	–	40

At various times after infection 1 ml samples of cells were labeled with 100 μCi of [5′-^{3}H]uridine (spec. act. 5 mCi/0.096 mg) for 2 min and the RNA was extracted according to [14]. T4 DNA strands were separated with poly(U,G) in CsCl equilibrium gradients following standard procedures [20] and controlled by hybridization with wild type T4 early (2–5 min) and late (48–53 min) [^{3}H]RNA as indicated in [9].

RNA–DNA hybridization was carried out in 0.2 ml of 2xSSC at 65°C for 6 h and analyzed [9]. Hybridization efficiency was at least 80%. The amount of radioactivity collected on filters in the absence of DNA strands was subtracted as background. All the experiments were done at least in duplicate. (- - -) indicates that the infection was carried out at 30°C and CAM was added 10 min after infection (line 2)

All the identified late proteins, missing at 42°C are present after temperature shift-down (data not shown).

3.4. *Reversibility of the tsp45 in the uncoupled system*

A possible explanation of these findings would be that there is a competition between DNA replication and late gene transcription for the intracellular p45 pool.

In order to decrease the number of p45 molecules involved in DNA replication we analyzed the messenger RNA produced in the system where the late gene expression takes place also in the absence of DNA replication [7].

E. coli B^E cells were infected at 42.6°C with the am4301-amH39X-amN130-tsCB53 (genes 43, 30, 46 and 45) multiple mutant phage, and the RNA synthesized after temperature shift-down in the presence and absence of protein synthesis was analyzed. Table 3 shows that also in this system, where no phage DNA replication takes place, the synthesis of late messenger RNA after temperature shift-down is completely dependent on concurrent protein synthesis.

4. Discussion

The results shown might indicate that the gene 45 product acts in vivo directly and independently

as a component of the T4 DNA replication apparatus and as a regulatory protein necessary for late transcription.

In fact, under conditions where DNA replication is required for late gene expression, after lowering the temperature the tsp45 made at 42.6°C allows the accumulation of concatemeric DNA molecules (fig.2), while late messenger RNA synthesis is blocked (table 3). Correspondingly, in the uncoupled system, where late messenger RNA synthesis does not require any DNA replication and where evidence was presented that the tsp45 is not involved in the DNA endonucleolytic cleavage of the pol⁻ lig⁻ exo⁻ phage mutant [19], the tsp45 made at 42.6°C after temperature shift-down does not allow the late gene expression to take place (table 3).

We can think then, that the role of tsp45 into late transcription does not depend on its role in DNA replication and there is no competition between DNA replication and late gene expression.

On other hand, since all ts45 mutants tested are genetically different, but show the same phenotype, it does not seem plausible in explaining our findings, to assume that the tsp45 has two sites: the first involved in phage DNA replication which would undergo thermal reactivation, and a second site involved in the control of late gene expression which would not be reactivated.

We favor a model where the two different roles shown by the tsp45 would depend on the different characteristics of the two mechanisms, DNA replication and late gene expression, in which the gene 45 product is involved. As a component of the DNA replication apparatus, the gene 45 product would be part of a multi-protein complex which allows thermal reactivation to take place.

On the other hand, the copies of the tsp45 that interact with the phage-modified host RNA-polymerase would be in a different configuration with respect to that described above. Such configuration would not allow renaturation of the gene 45 polypeptide upon temperature shift-down.

The reduced late gene expression observed in the presence of protein synthesis (fig.3) would be due then, to the tsp45 synthesized ex novo after shift-down at 30°C.

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