On the Direction of Translation of the T4 Deoxyribonucleic Acid Polymerase Gene in Vivo*

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Summary

A map of the structural gene for T4 DNA polymerase (gene \(\varepsilon\)) was constructed based on the immunological cross-reactivity and the molecular weights of the peptides generated by a series of amber mutants that map in this gene. The physical map deduced in this way was in good agreement with the genetic map determined on the basis of recombination frequencies. Analysis of the size and cross-reactivity of the amber peptides also demonstrated that the T4 DNA polymerase gene is translated in a counterclockwise direction along the circular genetic map of phage T4. This is the same direction that the lysozyme and rII genes of T4 are translated and is in the direction opposite to that of genes known to specify the structural components of the T4 capsid.

In the period immediately after infection of Escherichia coli with phage T4, a set of new proteins required for the synthesis of T4 DNA is induced. Since all of the messenger RNAs synthesized during this time are transcribed from only one of the two strands of the T4 DNA molecule (1), it should follow that the genes specifying these proteins are translated in the same direction. Thus far, the direction of translation has been examined for three of the early proteins, the products of genes \(\varepsilon\) (the phage-induced lysozyme), rIIA, and rIIB. In each instance translation was found to proceed in a counterclockwise direction along the circular T4 map (2, 3).

We have examined the direction of translation of another of the “early” genes, that responsible for the T4-induced DNA polymerase (gene \(\delta\)) (4, 5), by two independent methods and have found that, like the \(\varepsilon\) and rII genes, gene \(\delta\) is translated in a counterclockwise direction.

Materials and Methods

Strains and Media—Amber mutants of T4D, generously provided by Drs. R. S. Edgar (University of California, Santa Cruz) and J. W. Drake (University of Illinois), were propagated in E. coli CR63 according to the procedure of Edgar and Liebalsis (6). E. coli B served as the nonpermissive host for these mutants. H-broth (6) was used for the growth of uninfected cells, and a modified M-9 medium (7) was used when \(\varepsilon\)-labeled cells were required. All cultures were grown at 37°C.

Reagents—Carrier-free \(^{125}\)I (as NaI) and \(^{35}\)S (as HSO\(_4\)) were purchased from the New England Nuclear Company. Chloramphenicol was obtained from Parke-Davis, chloramine-T was from Baker Chemical Co. BRIJ was purchased from Atlas Chemical Industries. SDS was purchased from the Fisher and Porter Company. Salt-free lysozyme was obtained from the Worthington Biochemical Company.

The assay of T4 DNA polymerase and its purification from extracts of T4 am NS2 (gene \(\delta\))-infected E. coli B have been described previously (8). Rabbit antiserum against purified T4 DNA polymerase was very kindly prepared by Dr. Lawrence Levine. Sheep antiserum prepared against rabbit \(\gamma\)-globulin was purchased from the Grand Island Biological Company.

Preparation of Injected Cell Extracts— Cultures of E. coli B, grown to \(5 \times 10^8\) cells per ml, were infected with amber mutants in gene \(\delta\) (multiplicity of infection 5 to 8). The infection was terminated at 15 to 18 min by the addition of chloramphenicol (100 \(\mu\)g per ml) and NaN\(_3\) (0.01 M). The infected cultures were quickly chilled in a salt-ice bath, and the cells were collected by centrifugation at 10,000 \(\times g\) for 10 min. The cells could be stored indefinitely as frozen pellets. \(^{35}\)S-labeled infected-cell cultures were prepared in the same way except that a modified M-9 medium containing 3 \(\times 10^{-8}\) M NaSO\(_4\) was used. \(^{35}\)S HSO\(_4\) (80 to 300 \(\mu\)Ci per \(\mu\)mole) was added immediately after infection. BRIJ lysates of the infected cultures were prepared according to Swartz et al. (9).

Iodination of T4 DNA Polymerase—T4 DNA polymerase was labeled with \(^{125}\)I by the chemical iodination procedure of Hunter (10). The reaction mixture (0.1 ml) consisted of 0.15 M sodium phosphate (pH 7.5), 90 \(\mathrm{mg}\) (1.5 \(\mathrm{mCi}\)) of \(^{35}\)NaI, 35 \(\mu\)g of T4 DNA polymerase, and 200 \(\mu\)g of chloramine-T. After incubation at room temperature for 1 min, the reaction was terminated by the addition of sodium metabisulfite (2.4 mg per ml) and KI (5 mg per ml). The mixture was immediately applied to a column of Sephadex G-50 (1.2 \(\times\) 30 cm) which had

The abbreviations used are: BRIJ, BRIJ 58 (polyoxyethylene (20) cetyl ether); SDS, sodium dodecyl sulfate; CRM, cross-reacting material.
previously been equilibrated with 0.05 M Tris-HCl (pH 7.5), 0.1 M (NH₄)₂SO₄, 0.05% gelatin, and 1 mM dithiothreitol, and then eluted with the same buffer to separate the [³¹P]-labeled T4 DNA polymerase from unreacted NaI. Under these conditions, approximately 10 atoms of [³¹P] were bound per molecule of T4 DNA polymerase. Although the iodinated protein was no longer active as a polymerase, it nevertheless remained in an undenatured state after the iodination procedure as judged by the following criteria. (a) It retained its ability to bind rabbit antiserum prepared against purified T4 DNA polymerase. (b) The binding of [³¹P]-labeled polymerase to rabbit antiserum could be prevented by the addition of unlabeled polymerase. (c) [³¹P]-labeled and unlabeled polymerase had the same mobility during polyacrylamide gel electrophoresis in SDS and sedimented identically during neutral sucrose density gradient centrifugation.

Radioimmune Assay—Purified unlabeled T4 DNA polymerase inhibits the binding of [³¹P]-labeled polymerase to a constant amount of antiserum prepared against the purified enzyme. The extent of inhibition is proportional to unlabeled polymerase added (Fig. 1). A similar result was obtained with crude infected cell extracts. The extent of inhibition is a measure of the antigenically cross-reacting material in the extract. The standard reaction mixture for assay of CRM contained 0.01 M Tris-HCl (pH 7.5), 0.14 M NaCl, 0.5 mM MgSO₄, 0.15 mM CaCl₂, 0.1% gelatin, 0.03 μl of rabbit antipolymerase serum, 8000 cpm of [¹²⁵I]-labeled polymerase, and suitable amounts of cell extract. Incubation was at 5° for 40 to 50 hours, then 0.1 ml of 200-fold diluted normal rabbit γ-globulin were added. Incubation was continued for another 30 hours at 5°. The samples were then centrifuged for 45 min at 2500 rpm, and the [¹²⁵I] content of the precipitated antigen-antibody complexes was determined in a Nuclear Chicago 1085 γ-counter. The assay was effective within a range of approximately 0.01 to 1 μg of protein for purified enzyme and 10 to 1000 μg of protein for crude extracts (Fig. 1). One unit of CRM is defined as the amount of extract which produces a 50% inhibition of [³¹P]DNA polymerase-antipolymerase complex formation.

Polyacrylamide Gel Electrophoresis—Polyacrylamide gel electrophoresis of [³²S]labeled extracts in the presence of SDS was performed by the procedure of Reid and Bielski (11) for slab gels, modified to use the discontinuous buffer system of Laemmli (12). After electrophoresis, the slab gel was dried according to the method of Maizel (13) and autoradiographed (14). Samples for electrophoresis were dissolved in Buffer A, composed of 0.05 M Tris-HCl (pH 6.8), 2% SDS, 1% 2-mercaptoethanol, and 10% glycerol. They were then heated at 100° for 1 to 2 min.

[¹²⁵I]-Labeled λ and T4 phages were purified by CsCl density gradient centrifugation. The phage capsids, disrupted by heating to 100° for 1 to 2 min in Buffer A served as molecular weight standards (15, 16).

RESULTS

Cross-reacting Material in Extracts of E. coli B Infected with Amber Mutants in Gene 43—Although T4 DNA polymerase activity was low or undetectable in extracts of E. coli B infected with a variety of amber mutants in gene 43, material capable of cross-reacting with antibody to purified T4 polymerase was present (Table I). The level of CRM increased as the site of the amber mutation within gene 43 proceeded in the direction gene 62 to gene 42 (Fig. 2). A similar result was obtained when a protease inhibitor, phenylmethylsulfonyl fluoride (1 mM), was added to the infected-cell culture prior to the preparation of cell extracts. Since amber mutations cause premature polypeptide chain termination at the site of the amber codon (18) and if the assumption is made that reinitiation of protein synthesis at a site distal to the amber codon did not occur, then the gradient of

![Fig. 1. Linearity of radioimmune assay. The assay conditions are described under "Materials and Methods." Purified enzyme was prepared from T4 am N82 (gene 43)-infected Escherichia coli B (8), and the crude extract was prepared from T4 am N82-infected E. coli B treated with lysozyme and BRIJ (9).](image)

![Fig. 2. Gradient of CRM activity produced by amber mutants in gene 43. Assay of CRM is described under "Materials and Methods." The results given are the average of two experiments. The assignments of mutation sites and their map location are those of Allen et al. (21).](image)
Molecular weights of the products of T4 amber mutants in gene 43

Molecular weights of the T4 DNA polymerase and gene 43 amber peptides were determined by comparing their migration on SDS-polyacrylamide slab gels with the migration of the T4 and λ phage capsid proteins. The assignment of amber mutants is that of Allen et al. (21).

| Mutant     | Molecular weight (× 10⁻³) |
|------------|---------------------------|
| T4D        | 115                       |
| E4302      | 100                       |
| E4311      | 100                       |
| E4340      | 100                       |
| B22        | 90                        |
| E4315      | 90                        |
| E4317      | 76                        |
| E192       | 62                        |
| E4309      | 58                        |
| E4322      | 54                        |
| E4306      | 26                        |
| E4352      | 26                        |
| E4327      | 26                        |
| E4314      | 23                        |

Since, as noted above, the velocity of migration of a protein in SDS-polyacrylamide gel electrophoresis is inversely related to its molecular weight, the molecular weights of the gene 43 amber peptides could be estimated using the capsid proteins of phages λ and T4 as standards (Table II). The physical map of gene 43 constructed in this way is shown in Fig. 4. The genetically determined map (21) is included for comparison.

Precipitation of Gene 43 Amber Peptides by Antiserum to T4 DNA Polymerase—Although the amber peptides produced after infection with two of the gene 43 mutants, E4314 and E4327, showed no cross-reactivity in the radioimmune assay (Fig. 2), they were quantitatively precipitated by high levels of T4 DNA polymerase antiserum. Thus, when 30 μl of antiserum were added to ⁴⁵S-labeled extracts of E. coli B infected with B22, E4327, or E4314 (approximately 400 μg of protein), a molonecific precipitate was formed. The precipitate was separated from the supernatant fraction, and the redissolved precipitate and supernatant fractions were then analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 5,
The polypeptides synthesized after infection of E. coli B by amber mutants in T4 gene 43 are relatively stable and can be analyzed by radioimmune techniques as well as by SDS-polyacrylamide gel electrophoresis. Using two factor crosses, Allen et al. (21) determined the recombination frequencies between 49 amber mutants in gene 43, and located them at 11 distinct sites within the gene. We have chosen representative members at each of these sites and have shown by a radioimmune assay that the antigenic cross-reactivity of the amber peptides formed by these mutants increased as the mutation site approached the portion of gene 43 bordering on gene 42. This result demonstrates that the direction of translation of gene 43 proceeds counterclockwise, a result which agrees with the direction of translation determined for three other early genes (e, rIIA, and rIIB) and is consistent with that predicted by studies of early messenger RNA synthesis (1-3). It is also consistent with the finding of Nossal and Hershfield (22) that the polypeptide generated by amE22 which lacks the polymerase but retains the nuclease activity of the wild type enzyme has a molecular weight approximately 80% that of the wild type T4 polymerase (see Table II).

O'Donnell and Karem (23) have recently reported that double mutants in gene 43 that contain both the amE22 mutation and another amber mutation located clockwise to the B22 site do not make the exonuclease characteristic of B22 mutants, and hence have also concluded that gene 43 is translated in a counterclockwise direction. Amber mutants which map close to the section of gene 43 bordering on gene 42 (presumably the NH2 terminus of the T4 DNA polymerase) produced little if any CRM. Nevertheless, the amber peptides that were formed were identifiable by SDS-polyacrylamide gel electrophoresis and were precipitated by high concentrations of T4 DNA polymerase antiserum. They must therefore contain determinants recognizable by the antibody to the T4 DNA polymerase.

The amber peptides generated by the gene 43 mutants examined were well resolved on SDS-polyacrylamide gel electrophoresis because of their difference in molecular weight, a result which provided the basis for the construction of a physical map for gene 43. The physical map for gene 43 determined in this way was in good agreement with the map derived from studies of recombination frequencies between gene 43 mutants and was consistent with the order of mutations determined by the CRM assays. As expected, the frequency of recombination between two mutational sites depended upon the physical distance between these two sites; it was calculated to be approximately 0.2 to 0.3 per 1000 daltons of polypeptide synthesized. This value was constant throughout gene 43 and agrees well with the value reported by Beckendorf and Wilson (24) for the fine map of gene 34, one of the genes involved in the synthesis of the tail fiber of T4 phage. Unlike gene 34, which contains a region near its COOH terminus that gives an abnormally high frequency of recombination, gene 43 showed no significant recombination "hot spots," although mutants located near the COOH terminus were more readily obtained than those near the NH2 terminus (21).

It is interesting to note that gene 43 as defined by genetic recombination is about 15,000 to 20,000 daltons shorter at both termini than the physically determined map. In other words, mutants have not been isolated that map in regions closer than 15,000 to 20,000 daltons from the two ends of the polymerase gene as defined by its physical map. This result may be related to the procedure used for mutant selection. Thus all amber mutants of T4D were originally isolated on the basis of their suppressibility by E. coli CR63 which is a serine suppressor (26).

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REFERENCES
1. Guha, A., Szymbalski, W., Salser, W., Bolle, A., Goldscheik, E. P., and Pultizer, J. F. (1971) J. Mol. Biol. 59, 329–349
2. Streisinger, G., Emrich, J., Okada, Y., Tsugita, A., and Isoute, M. (1968) J. Mol. Biol. 31, 607–612
3. Chick, F. H. C., Barnett, L., Brenner, S., and Watts-Tobin, R. J. (1961) Nature 192, 1227-1232
4. DeWaard, A., Paul, A. V., and Lehman, I. R. (1965) Proc. Nat. Acad. Sci. U. S. A. 54, 1241
5. Warner, H. R., and Barnes, J. E. (1966) Virology 28, 100
6. Edgar, R. S., and Lielauks, I. (1964) Genetics 49, 649
7. Georgopoulos, C. P., Hendrix, R. W., Casjens, S. R., and Kaiser, A. D. (1972) J. Mol. Biol., in press
8. Huang, W. M., and Lehman, I. R. (1972) J. Biol. Chem. 247, 3139
9. Swartz, M. N., Nakamura, H., and Lehman, I. R. (1972) Virology 47, 538
10. Hunter, W. M. (1967) in Handbook of Experimental Immunology (Weir, D. M., ed.), p. 608, F. A. Davis Co., Philadelphia
11. Reid, M. S., and Bieleski, R. L. (1968) Anal. Biochem. 22, 374
12. Laemmli, U. K. (1970) Nature 227, 680-685
13. Maizel, J. V. (1971) in Methods in Virology V (Maramorosch, K., and Koprowski, H., eds) pp. 179-244, Academic Press, London
14. Fairbanks, G., Jr., Levinthal, C., and Reeder, R. H. (1965) Biochem. Biophys. Res. Commun. 20, 343
15. Casjens, S., Hohn, T., and Kaiser, A. D. (1970) Virology 42, 406
16. Dickson, R. C., Barnett, S. L., and Eisinger, F. A. (1970) J. Mol. Biol. 53, 461-474
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
18. Sarabhai, A. S., Stretton, A. O. W., Brenner, S., and Bolle, A. (1964) Nature 201, 13-17
19. Hosoda, J., and Levintal, C. (1968) Virology 34, 709
20. Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412
21. Allen, E. F., Albrecht, I., and Drake, J. W. (1970) Genetics 66, 187
22. Nossal, N. G., and Hershfield, M. S. (1971) J. Biol. Chem. 246, 5414-5425
23. O'Donnell, P. V., and Karim, J. D. (1972) J. Virol. 9, 990
24. Beckendorf, S. K., and Wilson, J. H. (1972) Virology, in press
25. Epstein, R. H., Bolle, A., Steinberg, C. M., Kellenberger, E., De La Tour, E. B., Chevalley, R., Eduar, R. S., Susman, M., Denhardt, G., and Lielauks, A. (1963) Cold Spring Harbor Symp. Quant. Biol. 27, 375