Genetic Mapping of the Amino-Terminal Domain of Bacteriophage T4 DNA Polymerase

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ABSTRACT

The DNA polymerase of bacteriophage T4 is a multifunctional enzyme that harbors DNA-binding, DNA-synthesizing and exonucleolytic activities. We have cloned in bacterial plasmids about 99% of the structural gene for this enzyme (T4 gene *43*). The gene was cloned in six contiguous 5′-terminal DNA fragments that defined seven intragenic mapping regions. *Escherichia coli* hosts harboring recombinant plasmids carrying the gene *43* subsegments were used in marker-rescue experiments that assigned a large number of *ts* and nonsense polymerase mutations to different physical domains of the structural gene. Conspicuously, only one missense mutation in a large collection of mutants mapped in the 5′-terminal 450 base-pair segment of the approximately 2700 base-pair gene. To test if this indicated a DNA polymerase domain that is relatively noncritical for biological activity, we mutagenized a recombinant plasmid carrying this 5′-terminal region and generated new conditional-lethal mutations that mapped therein. We identified five new *ts* sites, some having mutated at high frequency (nitrosoguanidine hot spots). New *ts* mutations were also isolated in phage genes *62* and *44*, which map upstream of gene *43* on the T4 chromosome. A preliminary examination of physiological consequences of the *ts* gene *43* mutations showed that they exhibit effects similar to those of *ts* lesions that map in other gene *43* segments: some were mutators, some derepressed gene *43* protein synthesis and they varied in the severity of their effects on T4-induced DNA synthesis at nonpermissive temperatures. The availability of the gene *43* clones should make it possible to isolate a variety of lesions that affect different activities of the T4 DNA polymerase and help to define the different domains of this multifunctional protein.

THE DNA polymerase of bacteriophage T4 is a multifunctional enzyme that plays a central and essential role in phage DNA replication. The biological activities of this enzyme reside in a single polypeptide chain of 100–110 kilodaltons (kD) (GOULIAN, LUCAS and KORNBERG 1968) that is encoded by phage gene *43* (DEWAARD, PAUL and LEHMAN 1965; WARNER and BARNES 1966) and that possesses two enzymatic activities: the DNA synthesizing activity (polymerase) which catalyzes extension of a nucleic acid primer from its 3′-hydroxyl end (5′→3′ DNA chain growth) in the presence of a template, deoxyribonucleoside triphosphates (dNTPs) and Mg^{2+} and an exonucleolytic activity that degrades DNA, particularly denatured species, in the 3′→5′ direction in the absence of deoxyribonucleotides (GOULIAN, LUCAS and KORNBERG 1968). Under *in vitro* conditions that are favorable for DNA synthesis, the 3′-exonuclease function of T4 DNA polymerase will also catalyze template-dependent nucleotide turnover (dNTP→dNMP) at the 3′-terminus of the growing DNA chain, *i.e.*, hydrolysis of newly incorporated nucleotides (GOULIAN, LUCAS and KORNBERG 1968; HERSHFIELD and NOSSAL 1972); this activity is more effective against misincorporated (mispaired) bases than against correctly inserted precursors and provides the T4 gene *43* protein with a proofreading function.

The abilities of the T4 gene *43* protein to select and edit precursors for DNA chain growth *in vivo* are major determinants in the control of the fidelity of phage DNA replication. Both mutagenic and antimutagenic missense mutations of this gene have been identified (SPEYER 1965; SPEYER, KARAM and LENNY 1966; DRAKE *et al.* 1969; REHA-KRANTZ and BESSMAN 1981; REHA-KRANTZ *et al.* 1986). In some cases, the altered enzymes encoded by gene *43* mutants have been isolated and shown by *in vitro* assay to differ from wild-type enzyme in their efficiencies of either precursor insertion (*i.e.*, base selection) or excision of misincorporated bases (*i.e.*, editing by the 3′-exonuclease) (for a recent review see SINHA and GOODMAN 1983). These polymerase-mediated controls over reading accuracy seem to depend on several important properties of this enzyme, including nucleotide sequence-dependent interactions with the DNA template, size and composition of the nucleotide pool, and interactions between the enzyme and other protein components of the DNA replication complex (for

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recent reviews and references, see NOSSAL and ALBERTS 1983 and KARAM, TROJANOWSKA and BAUCOM 1983). Some T4 replication proteins have been shown to stimulate the polymerase and exonuclease functions of the gene *43* product *in vitro* (VENKATESAN and NOSSAL 1982). In addition, the T4 polymerase regulates its own synthesis (RUSSEL 1973), possibly at the transcriptional level (KRISCH *et al.* 1977; MILLER *et. al.* 1981) and presumably by interacting with a specific segment of the T4 DNA that is designated for control of gene *43* transcription.

The capacities of the T4 gene *43* product for a variety of protein-DNA, protein-nucleotide and protein-protein interactions are probably reflections of the structural organization of this protein. Several observations suggest that different activities or interactions of the gene *43* protein are specified by different, albeit overlapping or cooperating, segments (domains) of the polypeptide chain. NOSSAL (1969) observed that an amber (*am*) nonsense protein fragment encoded by the T4 gene *43* mutant *43amB22* lacks DNA synthesizing activity but possesses a 3′-exonucleolytic activity similar to that of wild-type T4 DNA polymerase. The *B22* protein corresponds to the N-terminal 70–80% of wild-type T4 gene *43* protein and, unlike wild-type enzyme, it binds DNA poorly. Thus, it appears that the C-terminal 20–30% of the T4 DNA polymerase chain harbors determinants that are important for DNA-binding and polymerizing activity. Another possible indication of functional domains within the gene *43* polypeptide derives from the observed distribution of mutator and antimutator lesions within gene *43*. REHA-KRANTZ and BESSMAN (1981) found that *ts* gene *43* lesions that strongly affect mutation rates tend to map at two clusters within this gene and suggested that these clusters define polymerase-exonuclease active sites of the protein. Gene *43* mutations, however, often yield aberrant recombination frequencies in genetic crosses (ALLEN, ALBRECHT and DRAKE 1970) and, consequently, the available fine-structure genetic map for gene *43* cannot be adequately described in terms of physical distances. In this report, we describe the development of an experimental system for establishing a physical map for gene *43* that can be reliably correlated with the genetic map.

We cloned several subfragments of the T4 gene *43* region in bacterial plasmids and used these in simple and rapid marker-rescue tests that assigned a large number of known missense and nonsense gene *43* mutations to physically defined intragenic domains. The mapping studies revealed a paucity of *ts* lesions in the 5′-terminal 15–20% of the gene; however, we were able to isolate, *via* mutagenesis of recombinant plasmids, several new *ts* mutations that mapped within this segment of the gene. The biological properties of the new mutants implicate the NH_2-terminal 70-amino-acid domain of T4 DNA polymerase in the interactions underlying mutator effects and autogenous regulation. Potentially, the family of plasmids and experimental approaches we describe here will allow targeted and detailed genetic manipulation of the activity domains for this multifunctional phage-induced enzyme.

MATERIALS AND METHODS

Phage: Most of the T4 mutants we used came directly from R. S. EDGAR and W. B. WOOD (Caltech collection). Duplicate stocks of some of the *ts* gene *43* strains and the highly revertible *rIIB* mutant *rUV117* were kindly provided by J. W. DRAKE. The gene *43* UGA mutants *43N0943*, *43N1243*, and *43H4350* and the missense mutant *43ts1043* were isolated in this laboratory (CHAO, LEACH and KARAM 1977). Other *ts* mutants of T4 genes *43*, *62*, and *44* were isolated in the study to be described here.

Bacteria: *E. coli* K12 strain CR63, which carries the *am* suppressor su^+1 (*supD*, ser), served as the permissive host for T4 *am* mutants. *E. coli* K12 strain CAJ70(λ), which contains the UGA suppressor su^+9 (SAMBROOK, FAN and BRENNER 1967), was cured from its λ by heteroimmune superinfection with λ*hy b2* phage and the cured derivative was used in the preparation of lysates of the T4 UGA, *ts*, and *rII* mutants. CAJ70(λ) was used in experiments that measured the effects of T4 gene *43* mutations on reversion of *rIIBUV117*. *E. coli* K12 strain K802 [$hsdR^-$, $hsdM^+$, gal^-, met^-, $supE(su^+2, gln)$] (WOOD 1966) was obtained from N. MURRAY and used as host for cloning vectors and recombinant plasmids. *E. coli* B strain B^E (from L. GOLD) and a streptomycin-resistant derivative low in ribosomal ambiguity (isolated in this laboratory) were used as nonpermissive (sup^o) indicator strains for T4 *am* and UGA mutants.

Bacterial and phage growth and plating conditions: The media and conditions used to grow bacterial cultures and phage lysates were as described by KARAM and O'DONNELL (1973). The restrictive and permissive temperatures for *ts* mutants were 42° and 30°, respectively. Conditions for the radiolabeling of phage-induced proteins and their analysis by SDS-gel electrophoresis and autoradiography (Figure 1 and Table 1) are described elsewhere (CHAO, LEACH and KARAM 1977; KARAM, MCCULLEY and LEACH 1977; and HSU *et al.* 1987). We used the following protocol for measuring [^{3}H]thymidine incorporation. *E. coli* $B^E str^r$ was grown at 30° in M9S synthetic medium to 3×10^8 cells/ml and then aerated at 42° for 10 min before use in infections with T4 *ts* gene *43* mutants and wild-type phage. A prewarmed 0.1-ml aliquot of phage (3×10^8 plaque-forming units in M9S) was added to 0.1 ml (3×10^7) cells and the mixture was aerated at 42° for 20 min before isotope addition. The [^{3}H]thymidine (New England Nuclear catalog no. NET-027E) was preadjusted to a specific activity of 10 μCi ^{3}H per μg thymidine and used at a concentration of 12 μCi ^{3}H per ml of infection mixture. Isotope incorporation was allowed to occur for 10 min at 42° in the aerated culture before it was stopped by rapid chilling and concurrent addition of 5 ml of chilled 5% solution of trichloroacetic acid. A drop of 0.5% bovine serum albumin solution was used as carrier. Acid-precipitable material was allowed to accumulate for 20 min in an ice bath before the suspensions were filtered over glass-fiber discs (Schleicher & Schuell No. 25) and counted for ^{3}H in a toluene-based scintillator.

Construction of recombinant plasmids: The plasmid

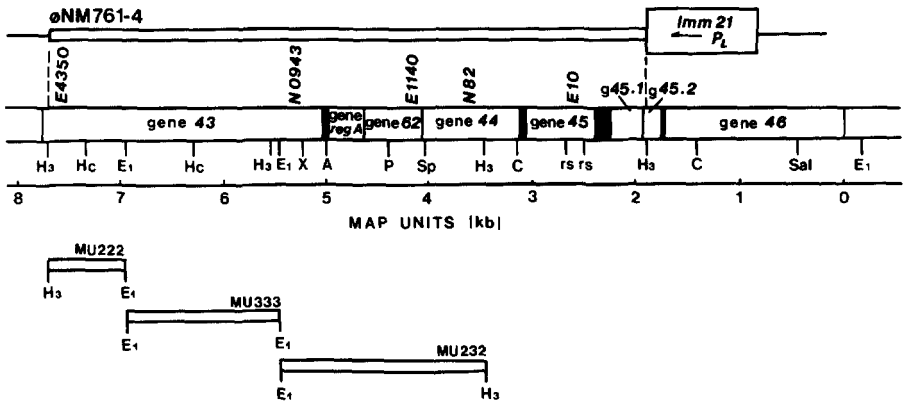

FIGURE 1.—Diagramatic representation of the T4 chromosomal sector that carries gene *43* and some of its upstream neighbors. The diagram also shows some restriction enzyme sites for this region and the boundaries of the T4 DNA insert (*Hin*dIII-*Hin*dIII fragment) in lambdoid clone NM761-4. The designations *H4350, N0943, E1140, N82,* and *E10* refer to T4 nonsense mutations that were shown to map at the indicated regions by marker-rescue tests. The bars labeled MU222, MU333, and MU232 refer to restriction fragments that were derived from NM761-4 and subcloned in the plasmid vector pBH20. The following abbreviations are used to designate restriction enzyme sites: H3 (*Hin*dIII), Hc(*Hin*cII), E1 (*Eco*RI), X (*Xho*I), A (*Ava*I), P (*Pst*I), Sp (*Sph*I), C (*Cla*I), rs (*Rsa*I), and Sal (*Sal*I).

cloning vectors we used were all derivatives of pBR322 and included pUC8 (VIEIRA and MESSING 1982) pBH20 (Itakura *et al.* 1977), and pGW7 (G. WILSON, N. E. Biolabs). These vectors contain inducible regulatory sequences that are not relevant to the studies discussed here. The source of T4 DNA for constructing plasmid clones of specific phage genome segments was the lambdoid recombinant phage NM761-4, which was provided by N. MURRAY and G. WILSON. A restriction/genetic map of the T4 DNA insert in phage NM761-4 is shown in Figure 1. Information for this map was derived from two types of analyses: the cloning and restriction mapping in pBH20 of *Hin*dIII and *Eco*RI endonuclease digestion fragments of NM761-4 DNA and genetic marker-rescue tests that localized the wild-type alleles for known *am* and *ts* sites of T4 genes 45, 44, 62 and 43. The methods for gene cloning and restriction enzyme mapping of plasmid clones were very similar to those described in MANIATIS, FRITSCH and SAMBROOK (1982). Marker-rescue tests utilized *E. coli* K802 cultures that either were preinfected (15–30 min at 37°) with phage NM761-4 or that contained a recombinant plasmid harboring a subfragment of NM761-4 DNA. About 0.05 ml of cells (at10^8/ml) were mixed with 0.05 ml T4 mutant phage (at 5 × 10^8/ml) and incubated at 30° for 30 min. The mixture was then spotted onto an *E. coli* $B^E str^r$ lawn and incubated either at 30° (for marker-rescue with T4 nonsense mutants) or at 42° (for T4 *ts* mutants). Cell lysis or the appearance of plaques in excess of what was observed in control spots was indicative of recombination (marker-rescue) between intracellular NM761-4 or plasmid and superinfecting T4 phage mutant. *E. coli* K802 cultures that were not preinfected with NM761-4 and cultures containing the cloning vectors without a T4 insert were used in control tests. Other recombinant plasmid constructions for use in fine-structure mapping of T4 gene *43* mutations are described in the RESULTS.

Conditions for nitrosoguanidine mutagenesis of recombinant plasmids and for isolation of *ts* T4 mutants: The methods we used were based on those described by MILLER (1972) and VÖLKER and SHOWE (1980). A recombinant plasmid named pMU232 (Figure 1) was introduced into *E. coli* CAJ70 by transformation and the plasmid-bearing cells were aerated at 30° in M9S medium containing 20 μg/ml ampicillin until grown to mid-log phase (3–5 × 10^8 cells/ml); small aliquots were withdrawn for verification of plasmid identity by agarose gel electrophoresis (MANIATIS, FRITSCH and SAMBROOK 1982) and by marker-rescue tests. The grown culture was harvested by centrifugation at 3000 × *g* for 5 min and resuspended in 0.1 M sodium citrate buffer (pH 5.5) at the original volume. This washing procedure was repeated once and the cell pellet was finally suspended in the same buffer containing 500 μg/ml *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma). After a 15-min incubation with mild shaking in a 30° water bath, the cells were pelleted by centrifugation, washed once in 0.1 M potassium phosphate buffer at pH 7.0, and then resuspended in M9S at one-half the original culture volume. The resuspended, mutagen-treated cells were aerated at 30° for 3 hr before being infected with T4 mutant *43N0943* (Figure 1) at a multiplicity of 3. Cell concentrations were estimated from microscope counts on samples of the cultures. After 20 min of infection, unadsorbed phage were removed by centrifugation at 5000 × *g* for 5 min and the infected cells were resuspended in M9S and aerated at 30° until lysed. A few drops of chloroform were added to complete lysis and the cell debris was removed by centrifugation at 10,000 × *g* for 10 min. A control lysate was prepared from a portion of the same pMU232-carrying CAJ70 culture that was subjected to all manipulations except for exposure to the mutagen. Under these experimental conditions, control lysates exhibited titers near 10^{11} and mutagenized lysates near 10^{10} plaque-forming units per ml when plated on *E. coli* CAJ70. The frequency of $43N0943^+$ recombinant plaques in the lysates was determined in platings on *E. coli* $B^E str^r$ (*sup*° host). The frequency of these 43^+ plaques was about 10% when pMU232 was present in the host, and did not appear to change with mutagenesis. In contrast, T4 *43N0943* phage lysates prepared on CAJ70 carrying the cloning vector without a T4 insert (*i.e.*, pBH20) exhibited a 43^+ "revertant" frequency of about 10^{-7} with unmutagenized cultures and this frequency increased about 4-fold as a consequence of the mutagenic treatment.

Screening for ts T4 mutants: The methods were similar to those described by EDGAR and LIELAUSIS (1964). Appropriate dilutions of phage lysates prepared as described above were plated on the *E. coli* $B^E str^r$ host, which scored only recombinant (or revertant) 43^+ progeny. Plaques began to appear on the bacterial lawns after about 5 hr of incubation at 30°. At this stage the plates were moved to a 42° incubator. After overnight incubation at the higher temper-

ature, *ts* T4 mutants appeared as small, sharp-edged plaques amid a variety of plaque morphologies. All minute plaques were picked with sterile toothpicks and stabbed into two *E. coli* $B^E str^r$ lawns that were subsequently incubated at 30° and 42°, respectively. Those plaques that failed to grow at 42° were picked from the 30° plate and purified by replating on the *E. coli* CAJ70 host, and lysates were prepared on CAJ70 from the purified plaques. Dilutions of these lysates were used in mixed-drop crosses on *E. coli* $B^E str^r$ lawns incubated at 42° to determine the number of different *ts* sites revealed by the mutagenesis. Representatives of the different sites were then used in marker-rescue tests to determine the locations of the *ts* lesions within the T4 DNA segments defined by cloned T4 DNA restriction fragments. About half of the *ts* mutants isolated in the nitrosoguanidine mutagenesis schemes described above mapped within the T4 MU232 DNA fragment. These mutants represented about 0.3% of all 43^+ recombinants. The frequency of *ts* T4 mutants of the MU232 region in lysates of unmutagenized controls was less than 0.005% of recombinants; we found none among 5000 recombinant plaques tested.

RESULTS

Construction of recombinant plasmids for mapping T4 gene *43* mutations: Figure 1 shows a genetic/restriction map of the T4 DNA segment carried by the λ*imm21*-T4 recombinant phage NM761-4. Marker-rescue tests had indicated that this phage harbored wild-type alleles to all of the known nonsense lesions of gene *43*, including its most distal C-terminal marker, the UGA mutation *43H4350*. NM761-4 also synthesized, under control of its *imm21* leftward promoter (P_L), a protein slightly larger in molecular size than normal, T4-induced DNA polymerase. This is shown in Figure 2. In addition, recent nucleotide sequencing information on T4 gene *43* (E. SPICER, personal communication), indicates that phage NM761-4 carries the coding information for all but nine C-terminal amino acids of T4 DNA polymerase in one continguous nucleotide sequence.

An *Eco*RI digest, a *Hin*dIII digest, and an *Eco*RI-*Hin*dIII double-digest were prepared from purified NM761-4 DNA and the resulting fragments were cloned into appropriately digested pBH20 cloning vector. This yielded several recombinant plasmids, including some containing the T4 DNA segments designated MU232, MU333 and MU222 in Figure 1. Clones of subfragments of these were subsequently constructed in pUC8, pGW7 and pBH20, ultimately yielding a family of recombinant plasmids that defined the seven gene *43* mapping regions that are shown in Figure 3. As will be discussed below, the existence of gene *43* mapping segment G (Figure 3) is inferred only from DNA studies; there are no known mutations that map in this portion of gene *43*.

Physical mapping of T4 gene *43* mutants: The relative order and genetic properties of a large number of T4 gene *43* lesions have been determined in a variety of genetic and biochemical studies (ALLEN, ALBRECHT and DRAKE 1970; O'DONNELL and KARAM 1972; KARAM and O'DONNELL 1973; REHA-KRANTZ and BESSMAN 1981; HUANG and LEHMAN 1972; NOSSAL and HERSHFIELD 1971; REHA-KRANTZ *et al.* 1986). By combining information from these studies with the results of marker-rescue tests that assigned *ts* and nonsense gene *43* lesions to defined gene *43* DNA subsegments (MATERIALS AND METHODS), we arrived at the map shown in Figure 4. Several features of this map are worth noting:

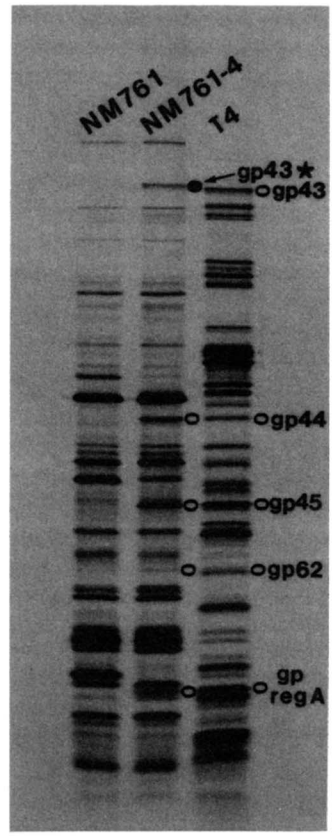

FIGURE 2.—An autoradiogram showing the proteins synthesized by lambdoid recombinant phage NM761-4 in infected *E. coli*. Note that this phage synthesizes a protein (gp43* band) that is slightly larger than normal T4 gene *43* protein (gp43 band, T4 lane). Phage NM761 is the lambdoid vector (WILSON and MURRAY 1979) that was used for cloning the T4 DNA by G. WILSON and N. MURRAY (personal communication). The methods that were used for ^{35}S-labeling of phage-induced proteins and for their analysis by SDS-gel electrophoresis and autoradiography are described elsewhere (HSU *et al.* 1987).

1. The majority of known T4 *ts* gene *43* mutations cluster in two contiguous 3′-terminal DNA fragments (segments E and F) that encompass about one-fourth of the gene.

2. All the mutations could be assigned to one or another of the six subcloned gene *43* fragments, *i.e.*, there have apparently been no lesions found for the 3′-terminal 27 base-pair segment G.

3. Only a small number of lesions mapped in the two 5′-terminal segments A and B (one missense lesion in the 197 base-pair A segment, *i.e.*, site *A58*, and two

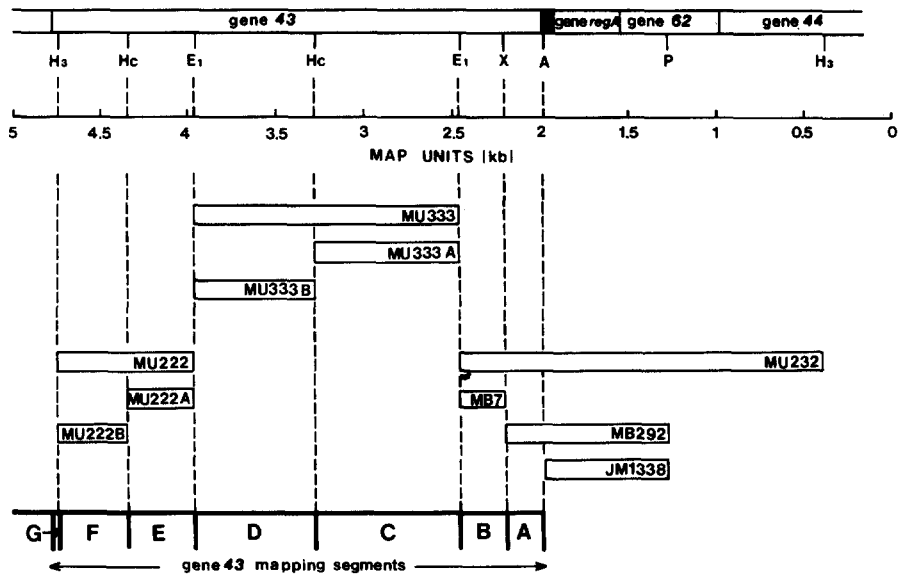

FIGURE 3.—A physical map showing the boundaries of the T4 DNA fragments used to define the gene *43* mapping segments for this study. All fragments were cloned in pBR322-derived vectors. MU222, MU333, MU232, and MB7 were cloned in pBH20. MU222A, MU222B, MU333A, and MU333B were cloned in pUC8. MB292 and JM1338 were cloned in PGW7. Recombinant plasmids were placed in *E. coli* K802 (*supE*) for mapping *ts* and *am* gene *43* mutants and in *E. coli* CAJ70 (*su*$^+$ UGA) for mapping UGA mutants. The abbreviations used to designate restriction enzyme sites are the same as in Figure 1.

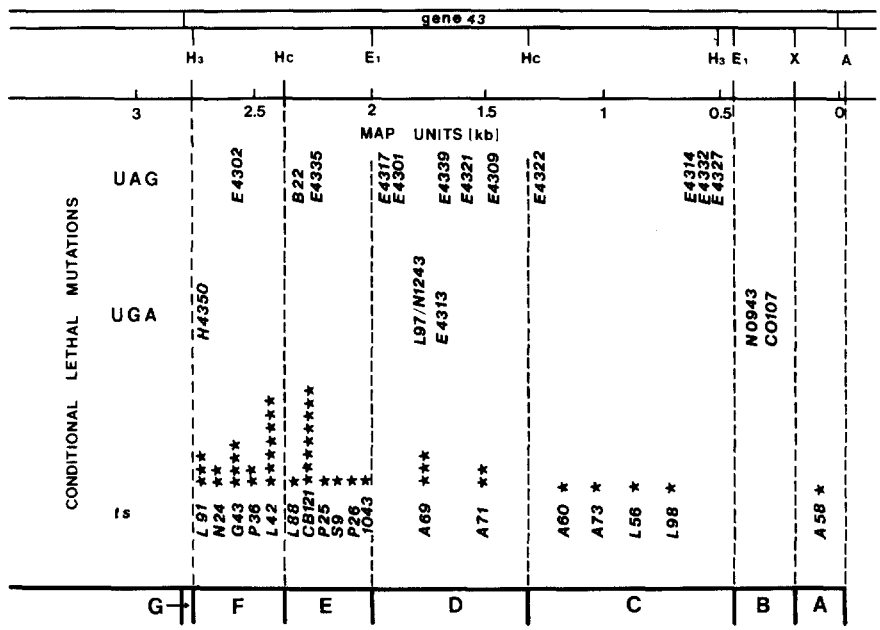

FIGURE 4.—A genetic/physical map of T4 gene *43* showing the relative positions of *ts* and nonsense mutations. The genetic order of *ts* sites relative to one another and to *am* (UAG) sites is largely based on reports in the literature (ALLEN, ALBRECHT and DRAKE 1970; REHA-KRANTZ and BESSMAN 1981; REHA-KRANTZ *et al.* 1986) and on our unpublished work. The *ts* mutation *1043* was isolated in this laboratory (CHAO, LEACH and KARAM 1977) and its order relative to other markers was determined by 3- and 4-factor crosses. The asterisks (*) mark the number of occurrences of mutations (ALLEN, ALBRECHT and DRAKE 1970). The relative order of most of the nonsense (UAG and UGA) lesions is based on genetic data (ALLEN, ALBRECHT and DRAKE 1970; KARAM and O'DONNELL 1973) and on measurements of the sizes of protein fragments that are generated by the mutants in *sup*^{e} hosts (HUANG and LEHMAN 1972; and our unpublished results). The order for UGA sites *N0943* and *C0107* relative to each other is based on 2- and 3-factor crosses. The mutation *L97* had previously been classified as *ts* but is a leaky UGA mutation (see text). Assignments of mutant sites to the different physical segments A, B, C, D, E and F are based on the results of marker-rescue tests (MATERIALS AND METHODS). Note the clustering of *ts* mutations in the 3′-terminal segments of the gene.

UGA mutations in the 250 base-pair B segment, *i.e.*, sites *N0943* and *C0107*.

4. Amber mutant sites seemed to be evenly distributed within the internal segments (C, D, E and F) of the gene.

We also should mention that we encountered some difficulties and inconsistencies in map assignments for a few of the *ts* gene *43* mutant phage strains examined. In most of these cases, published information about the order of a *ts* marker relative to other mutations

could not be confirmed by the physical mapping. We were unable to trace the sources of the mixups and can only caution investigators about the use of *ts* gene *43* mutants that bear certain designations. Briefly, our concerns are based on the following observations:

1. Previous studies had assigned the *ts* gene *43* mutations *A68*, *A69*, and *L107* to different sites (ALLEN, ALBRECHT and DRAKE 1970). In our analyses of phage strains from both our collection and the DRAKE collection, we observed no recombination in crosses between strains carrying these designations. So, in Figure 4, we use only the designation *A69* to describe the site, which maps in physical segment D.

2. We obtained another sample of *43tsL107* from W. B. WOOD's library at the University of Colorado (Caltech parent collection) but were unable to find *ts* plaques in it. Instead, we found a previously unrecognized UGA mutation that mapped in segment B, upstream of *N0943* (Figure 4). We have named this mutation *CO107*.

3. The mutation *A58* exhibits poor growth at both 30° and 42° with no apparent temperature-dependent defect. It may simply be a leaky missense mutation.

4. The mutation *L97*, previously designated as *ts*, is a UGA mutation that exhibits very leaky growth at 30° on *sup°* hosts. It is much less leaky at 42° and fails to grow at any temperature when plated on streptomycin-resistant *sup°* hosts that have diminished levels of ribosomal ambiguity (*e.g.*, as described by KARAM and O'DONNELL 1973). *L97* stocks from all sources tested (our collection, DRAKE's collection and a parent collection reissue) showed similar growth characteristics and failed to recombine with *N1243* (Figure 4), a spontaneously generated gene *43* UGA mutation that was isolated as a suppressor for a T4 gene *42* defect (CHAO, LEACH and KARAM 1977). When grown in *sup°* hosts, T4 *43tsL97* (and *43N1243*) stocks generated a gene *43* protein nonsense fragment of about 90 kD that could be identified by gel-electrophoretic assays (results not shown, but see CHAO, LEACH and KARAM 1977).

5. The *ts* mutation *L56*, a well known mutator, mapped in segment C (Figure 4). Genetic studies (ALLEN, ALBRECHT and DRAKE 1970) had placed it between *tsP26* (segmentE) and the mutation *L97* (segment D). We observed that some stocks of T4 *43tsL56* carried an additional, partially *ts* mutation in gene *43*. Such secondary mutation(s) may occur readily in mutator stocks and could be responsible for the differences in map assignments for the *L56* marker. The T4 *43tsL56* stock we used in other aspects of our work (discussed later) did not contain this secondary lesion and exhibited mutator effects similar to those that have been ascribed to this mutator in other studies (SPEYER, KARAM and LENNY 1966; DRAKE *et al.* 1969).

Isolation of 5′-terminal *ts* T4 gene *43* mutants: The observed rarity of conditional-lethal missense mutations in the 5′-terminal 450 base-pair portion of T4 gene *43* and the general skewing in distribution of such randomly isolated lesions towards the 3′-terminal third of the gene (Figure 4), raise the possibility that amino-acid replacements in the amino-terminal domain of T4 DNA polymerase are less detrimental to biological activity of the enzyme than are replacements of more internal or C-terminal residues. Such situations exist, *e.g.*, the *lacZ* gene product (β-galactosidase) of *E. coli* (MÜLLER-HILL and KANIA 1974) and the *rIIB* gene product of T4 (CRICK *et al.* 1961; BENZER and CHAMPE 1962). In the experiments to be described below, however, we found that many missense mutations can be readily isolated in the N-terminal segment of the T4 DNA polymerase and that these exhibit effects on biological activity and phage viability similar to those of mutations in other segments of the gene.

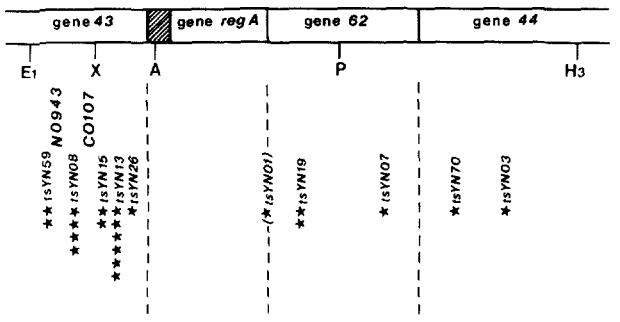

FIGURE 5.—Map distribution and relative frequencies of *ts* T4 mutations induced by nitrosoguanidine mutagenesis of a recombinant plasmid carrying the MU232 T4 DNA fragment diagrammed in Figure 1. The mutations were generated and isolated as described in the MATERIALS AND METHODS. Marker-rescue tests established locations of mutant sites to physical segments and 2- and 3-factor crosses determined their order relative to one another and to the UGA mutations *N0943* and *CO107*. The gene *43* mutation *A58* (Figure 4) maps within the A(*Ava*I)–X(*Xho*I) interval (*i.e.*, segment A, Figure 4) as determined by marker-rescue tests, but its order relative to the newly isolated *ts* markers in this interval could not be determined in phage crosses because of its high degree of leakiness.

The recombinant plasmid pMU232 contains gene *43* segments A and B as well as T4 genes *regA* and *62* and a 3′-terminal portion of gene *44* (Figure 3). *E. coli* CAJ70 (*su*$^+$UGA) cultures harboring pMU232 were mutagenized with nitrosoguanidine and then were used to grow a lysate of the UGA mutant *43N0943*, which maps in the B segment of gene *43* (Figure 4). The lysate was plated for UGA$^+$ recombinants under conditions that screened for *ts* mutants (MATERIALS AND METHODS). This led to the identification of ten new conditional lethal *ts* mutant sites within the T4 DNA segment defined by MU232. The distribution of these sites is diagrammed in Figure 5. As expected, most of the mutations clustered around the *43N0943* marker used for selecting recombinants. Five new *ts* sites were identified within the gene *43*

portion of MU232, three in segment A and two in segment B. In addition, we obtained *ts* lesions in the 3′-terminal segment of gene *44* (two new sites), in gene *62* (two sites, none having been isolated previously), and in a region near the junction between genes *62* and *regA* (mutant *tsYN01*, which has not yet been mapped precisely). The properties of the lesions that map outside gene *43* will be described elsewhere. Because of the relative ease with which the *ts* gene *43* mutations were induced, we propose that the amino-acid sequence in the NH_2-terminal region of T4 DNA polymerase is essential for determining a biologically active structure for this enzyme. The mutations *tsYN15*, *tsYN13* and *tsYN26* are within 70 residues of the amino-terminus of the 896-amino-acid polymerase.

Properties of N-terminal *ts* T4 DNA polmerase mutants: In view of the results described in the previous section, we suspect that the skewed map distribution of the Caltech collection of *ts* gene *43* mutations (Figure 4) reflects the specificity of the mutagens that generated them, *i.e.*, mostly bromodeoxyuridine hot spots (EDGAR and LIELAUSIS 1964). Similarly, the newly isolated *43tsYN* family of mutants may represent nitrosoguanidine hot spotting. It is possible, however, that N-terminal and C-terminal lesions affect different polymerase functions. We conducted a preliminary examination of this possibility by assaying representatives of the two sets of mutants for three biological properties: mutator/antimutator effects, autogenous derepression of gene *43* protein synthesis and severity of the block in DNA synthesis at a nonpermissive temperature. Some results of our analyses are summarized in Table 1. There appeared to be no relationship between map positions of *ts* mutations and the phenotypes they exhibited. Both sets of mutants included representatives that were leaky (incomplete inhibition of DNA synthesis at 42°), were altered in autogenous repression and that affected phage mutation rates. Two tentative conclusions may be drawn from these results: the N-terminal domain of T4 DNA polymerase plays an integral role in the functioning of this enzyme as a DNA binding protein and as a mediator of fidelity in DNA replication, and the *ts* phenotype may not be sufficiently discriminating to differentiate between separate functional domains on the T4 DNA polymerase molecule.

TABLE 1

Properties of N-terminal *ts* T4 DNA polymerase mutants

T4 gene *43* allele[a]	[^{3}H]Thymidine incorporation at 42° (% of wild-type)[b]	gp43 synthesis at 30°[c]	-Fold increase in frequency of	
			rIIBUV117 reversion[d]	Mutation to acriflavin resistance[e]
tsYN26	44	Repressed	5.7	0.5
tsYN13	6	Derepressed	5.0	1.2
tsYN15	33	Repressed	4.7	8.2
tsYN08	<1	Repressed	1.8	6.0
tsYN59	13	Derepressed	22	11
tsL56	<1	Repressed	3	120
tsCB121	<1	Derepressed	8	6.4
tsL88	<1	Derepressed	3.2	11
tsL91	6	Derepressed	2.3	2.4
Wild type	100	Repressed	1	1

[a] Genetic map locations of the *ts* gene *43* alleles used are shown in Figures 4 and 5.

[b] [^{3}H]Thymidine incorporation into trichloroacetic acid precipitable material was measured in infected *E. coli* $B^E str^r$ cultures (MATERIALS AND METHODS). Under these conditions, the wild-type infection yielded 1.8×10^5 cpm $^3H/10^7$ infected cells (100% value in the table).

[c] The levels of synthesis of gene *43* protein (gp43) were measured by SDS-gel electrophoretic assays as described in the MATERIALS AND METHODS.

[d] Reversion frequencies of *rIIBUV117* to *rII*$^+$ were measured in platings on *E. coli* CAJ70 and CAJ70(λ) of the single *rIIBUV117* mutant (wild-type gene *43* allele) and of *rIIBUV117/ts* gene *43* double mutants. Three independently prepared phage lysates (from single plaques) were used for each determination. The control value for reversion of the *rII* mutant (wild-type gene *43* allele) was 33×10^{-7}. Values for different lysates did not vary by more than 3-fold within each group, except for *tsL91* and *tsYN08* doubles with *rIIBUV117*. In each of these two cases, one of the three lysates gave a very high value (>10-fold higher than the values for the other two lysates); these "jackpot" values were not used in the calculations.

[e] Three lysates of each of the single *ts* gene *43* mutants and wild-type phage were used to measure mutation frequencies to acriflavin-resistance according to the method described by REHA-KRANTZ and LAMBERT (1985). The mean frequency of acriflavin-resistant mutants in the T4 wild-type control was 8.7×10^{-6}.

DISCUSSION

The T4 DNA polymerase offers an attractive but challenging model system for evaluating the utility of genetic approaches in localizing the biological activity domains of multifunctional proteins. On the one hand, the activities of this enzyme can be described by simple assays both *in vitro* (*e.g.*, DNA binding, synthesis and degradation) and *in vivo* (*e.g.*, mutagenic potential, autogenous repression and DNA precursor incorporation). On the other hand, the protein participates in several processes (replication, recombination, repair and autogenous regulation) that are probably serviced by different multiprotein complexes, the compositions of which cannot yet be clearly defined (for reviews see MOSIG 1983 and KARAM, TROJANOWSKA and BAUCOM 1983). A missense mutation in the T4 gene *43* product could result in pleiotropic effects that complicate the interpretation of biological consequences in terms of enzyme structure alterations. Another problem that is encountered in the genetic analysis of T4 DNA polymerase is related to the role of this enzyme in recombination. In their studies on developing a fine-structure map for gene *43*, ALLEN, ALBRECHT and DRAKE (1970) recognized that crosses between phage mutants defective in this gene often yield anomalous recombination frequencies that make it difficult to translate genetic data into intragenic physical distances. The approach we used

here side-steps this potential source of anomalies by relying on qualitative, rather than quantitative, measurements of recombination between T4 genetic segments. Mapping by marker-rescue, in which mutant sequences on a T4 genome are replaced by wild-type sequences from cloned DNA, is analogous to deletion mapping. A finer-structure genetic/physical map of T4 gene *43* than we presented here (Figure 4) can ultimately be developed *via* two approaches: the further subcloning of the gene *43* restriction fragments we described and direct nucleic acid sequence determinations on the mutants. The primary structure of T4 gene *43* has recently been determined by using some of the clones we described (E. SPICER, personal communication), and it should be possible to design the necessary primers for sequencing (at the genomic or RNA level) any of the lesions we mapped in this work. The results we presented (Figure 4) also provide a new outlook on the clustering of randomly isolated gene *43* mutations. A large number of the *ts* gene *43* mutations that had been isolated previous to this work were observed to cluster in two 3′-terminal segments (E and F, Figure 4) representing about one-fourth of the size of this gene. We do not believe that this clustering is indicative of a specific functional domain within the corresponding portion of T4 DNA polymerase. Although some of the *ts* lesions that mapped in segments E and F (Figure 4) had occurred spontaneously (*e.g.*, S9 and *1043*), most of the others were generated by bromodeoxyuridine mutagenesis (Edgar and Lielausis 1964), and it is possible that the two 3′-terminal gene *43* fragments include "hot spots" for this mutagen. The ease with which we were able to isolate *ts* gene *43* mutations in segments far removed from the 3′-terminal clusters (Figure 5) underscores the caution with which we interpret the biological implications of these clusters.

In attempting to use genetic lesions as tools to understand the structural organization of T4 DNA polymerase, it is probably instructive to take into consideration the similarities and differences that this enzyme exhibits when compared to other DNA replication polymerases. As a family of enzymes, DNA polymerases display a wide array of molecular forms, including single-subunit species in prokaryotes and multisubunit species in both prokaryotes and eukaryotes (KORNBERG 1980, 1982). The T4-induced enzyme (gene *43*) represents one of the simpler forms. It is a single polypeptide chain consisting of about 900 amino acids and possessing two activities that are crucial for DNA replication: the template-directed DNA synthesizing activity (polymerase) and the DNA proofreading function (3′-exonuclease) (GOULIAN, LUCAS and KORNBERG 1968). It is the only DNA polymerase known to be required for T4 DNA replication. In contrast, *E. coli* utilizes at least two DNA polymerases in its replication, pol I and pol III; a third enzyme, pol II has not been implicated in any physiological function. Of the three *E. coli* polymerases, pol III resembles T4 DNA polymerase the most in biological properties. *E. coli* pol I resembles T4 DNA polymerase in some respects: it is a single polypeptide of about the same size as the T4 gene *43* product and possesses polymerase and 3′-exonuclease activities in the same molecule (KORNBERG 1980). The proofreading activity of the T4 enzyme, however, is much more active than that of the bacterial enzyme (HUANG and LEHMAN 1972) and pol I harbors an additional exonucleolytic activity (5′-exonuclease) that the phage enzyme does not have. Biologically, pol I and T4 DNA polymerase appear to perform different, although overlapping, roles in replication. *E. coli* pol I is used to remove RNA primers from initiated "Okazaki" DNA fragments (*via* its 5′-exonuclease activity) and to close the gaps between the fragments *via* its DNA synthesizing activity. The T4 enzyme, on the other hand, appears to have no role in RNA primer removal, a function that may be performed by a phage-induced ribonuclease, RNase H (see NOSSAL and ALBERTS 1983). In addition, the T4 gene *43* product is a major determinant of reading accuracy in DNA replication, whereas the *in vivo* role of pol I in replication fidelity is unclear. In this regard, the functional analogue to T4 DNA polymerase in *E. coli* is the DNA polymerase III. This bacterial enzyme has been isolated in several forms: pol III holoenzyme, pol III*, pol III′, and pol III core enzyme (for a review see MCHENRY 1985). The core enzyme, or the minimal isolated form that exhibits catalytic activity, consists of three subunits: α (140 kD), ε (27 kD) and θ (10 kD). Subunit α is the product of the *E. coli dnaE* gene (WELCH and MCHENRY 1982) and possesses some DNA polymerase activity by itself (SPANOS *et al.* 1981) Subunit ϵ is the product of the *dnaQ* (*mutD*) gene of *E. coli* and carries determinants for the 3′-exonuclease activity (SCHEUERMANN *et al.* 1983; SCHEUERMANN and ECHOLS 1984; ECHOLS, LU, and BURGERS 1983). The biological significance of the *mutD/dnaQ* gene in control of mutation rates in *E. coli* is well established (DEGNEN and COX 1974; COX and HORNER 1983) and ϵ protein isolated from mutator *dnaQ* (or *mutD*) strains is defective in 3′-exonuclease activity and proofreading *in vitro* (SCHEUERMANN and ECHOLS 1984). Thus, in contrast to T4 DNA polymerase, the polymerase and 3′-exonuclease activities of *E. coli* pol III are specified by separate polypeptide chains. The role of the θ subunit in pol III is still unknown, whereas it is clear that α and ϵ cooperate in maintaining a high fidelity of DNA synthesis *in vitro* (MAKI *et al.* 1986). In view of the observation by NOSSAL (1969) that a polymeraseless polypeptide fragment of gene *43* protein harbors 3′-exonuclease activ-

ity, it seems possible that the complete T4 DNA polymerase molecule is organized into discrete activity domains that can be mapped via differential effects of mutations in this gene on the various activities of its protein product. Like *E. coli* pol III core enzyme, T4 DNA polymerase is not highly processive on DNA-templates *in vitro* and requires interactions with auxiliary proteins in order to replicate natural templates *in vitro* at rates resembling those observed *in vivo*. Pol III holoenzyme possesses such properties. In T4, processivity is aided by the presence of the gene *44* and *62* proteins, which act as a complex that harbors DNA-dependent ATP/dATPase activity (NOSSAL AND ALBERTS 1983). In *E. coli*, the product of the *dnaN* gene has been implicated in increased processivity of certain pol III preparations (pol III* and holoenzyme), although no coexisting hydrolytic activity for ATP or dATP was observed (MAKI *et al.* 1986). In addition to containing *dnaN* protein (37 kD, also termed the β subunit), pol III holoenzyme harbors the *dnaZ* protein (52 kD, γ subunit), *dnaX* protein (32 KD δ subunit) and *dnaX-Z* protein dimer (τ subunit) as well as other uncharacterized polypeptides (MCHENRY and CROW 1979). Recently, it was observed that τ subunit caused pol III to dimerize, a molecular transition that may be essential for coordinating leading-strand with lagging-strand synthesis during DNA replication (MCHENRY 1982, 1985). While isolated T4 DNA polymerase is simpler in structure than pol III core enzyme, it is probably capable of the same type of interactions (albeit perhaps more weakly) as those that are physically demonstrable for the bacterial enzyme. Carefully designed protein isolation schemes, as well as genetic assays, implicate the T4 DNA polymerase in specific interactions with several T4-induced replication proteins including the gene *45-44-62* protein complex (NOSSAL and ALBERTS 1983), gene *32* single-strand-binding protein (HUBERMAN, KORNBERG and ALBERTS 1971) and the nucleotide biosynthesis enzymes which couple phage DNA replication to precursor pools (reviewed by MATHEWS and ALLEN 1983). An understanding of the structural organization of the single-polypeptide gene *43* protein should yield insights about the amino-acid domains within the molecule that participate in or direct the assembly of functional replication forms of T4 DNA polymerase.

Finally, we should point out that the work we described in this report demonstrates the feasibility of targeting segments of the T4 gene *43* product for mutagenesis and for selection of mutations with specific phenotypes. We utilized temperature-sensitivity because of the ease with which this phenotype can be scored for. It should be possible to select for a variety of additional types of lesions that affect such gene *43*-associated phenotypes as suppression of defects in related T4 DNA replication genes (*e.g.*, CHAO, LEACH and KARAM 1977), mutator and antimutator activity (*e.g.*, REHA-KRANTZ and BESSMAN 1981; REHA-KRANTZ *et al.* 1986), failure to grow on OptA hosts (GAUSS, DOHERTY and GOLD 1983) and intragenic suppression of conditional-lethal mutations. Also, the selection for suppressible nonsense (UAA, UAG and UGA) gene *43* mutations could offer many opportunities to compare the biological and enzymological consequences of selectively substituting more than one type of amino acid per defined gene *43* protein site *via* tRNA suppression. We can look forward to describing many T4 gene *43* lesions in interesting biological terms that can be correlated to primary structure alterations at both the DNA and protein levels. Ultimately, however, such information will need to be complemented with information from direct analyses of protein secondary and tertiary structure.

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