

MUTATIONS IN BACTERIOPHAGE T4 GENOME

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Summary. – Bacteriophage (phage) T4 belonging to T-even phages is one of the best known phages with a completely deciphered genome sequence. As a model of living systems, T4 phage has many technical advantages. It can be very easily grown in large quantities, manipulated by classical genetics, and engineered by site-directed mutagenesis. Many substances have been first tested for mutagenicity in T-even phages. The results of these tests were very often applicable to higher organisms due to similar mechanisms of mutagenesis. T4 phage is also important in phage therapy, which represents an alternative treatment of bacterial infections since the bacterial resistance to antibiotics has become a serious medical problem. The site-directed mutagenesis is a method that enables to introduce mutations which can influence phage affinity to bacteria and can be a practical technique for enriching phage collections and for widening specificity of phages for new bacterial strains now insensitive to phage therapy.

Key words: bacteriophage T4; phage therapy; mutation; mutagen; site-directed mutagenesis

Introduction

Phage therapy has been developed at the beginning of the twentieth century, yielding interesting results, but then, with the rush toward antibiotics in the forties, it was forgotten (Kutter, 1997). Nowadays, even though antibiotic-resistant bacteria are not such a burgeoning threat, the phage therapy is still appealing. Antibiotics involve certain risks. They kill a wide range of microorganisms and rid the human or animal host not just of harmful bacteria but of useful ones as well. Antibiotics can also cause allergies, intestinal disorders and yeast infections. None of these problems apply to phages (Radetsky, 1996). As phages may upregulate immune functions in humans, such treatment may be of particular interest in patients with primary and secondary immunodeficiency syndrome (Weber-Dąbrowska *et al.*, 2001).

The promising effects of therapies, which make use of natural phages caused great interest among the people

engaged in this field of medicine. Recently, several reviews on phage therapy have appeared (Duckworth and Gulig, 2002; Das, 2001; Pirisi, 2000; Sulakvelidze *et al.*, 2002; Summers, 2001; Weber-Dąbrowska *et al.*, 2000). In addition, a comprehensive information about the current status and perspectives of this therapy can be found on web pages (<http://surfer.iitd.pan.wroc.pl/phages/phages.html> and <http://www.evergreen.edu/phage/relatedarticles.html>).

As recently pointed out by Kaplan (Kaplan, 2002), phages could also be used in food production and packaging. Evidence is also accumulating that the phage therapy may also be applied to animals e.g. fish (Nakai and Park, 2002) or poultry (Huff *et al.*, 2002). Therefore, the phage therapy may regain its role in the treatment not only of human but also of veterinary infectious diseases.

Some new solutions of the problems connected with the phage therapy are needed, e.g. a modification of phage specificity and virulence, increasing the ability of long-term circulation of phages in mammals, and elimination of the possibility to establish lysogeny in the course of the treatment (Carlton, 1999). Phages can be isolated from various sources in the environment, but the possibilities of predicting their properties are obviously limited. An alternative method for enriching the phage collection is mutagenesis, which offers

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Abbreviations: 3D = three-dimensional; ORF = open reading frame

many possibilities for manipulation. Genetic engineering techniques enable introducing appropriate nucleotide patterns into phage genome and obtaining phages with desired properties.

Phage T4, which infects *Escherichia coli* and closely related *Shigella* species, is one of the best known phages with a completely sequenced genome (Kutter *et al.*, 1994). T4 phage began to serve as a model for the understanding of living systems almost fifty years ago, when the major experimental tools available were just radioisotopes and centrifuges. At present, in the age of recombinant DNA technologies and complete genome DNA sequencing, T4 phage is still very important in studying fundamental processes in cells.

As a model of living systems, T4 phage has many technical advantages besides its simplicity. It can be very easily grown in large quantities required by biochemists, manipulated by classical genetics, and engineered by site-directed mutagenesis. By using combinations of the above techniques it is possible (i) to assemble protein machines that are responsible for a particular phage function and (ii) to mutate each gene that contributes to this machinery. Thereafter the machine function can be relatively easily deciphered by combined *in vivo* and *in vitro* studies (Alberts, 1994).

T4 phage is also important for the phage therapy, which has been introduced as an alternative treatment of infectious bacterial diseases of humans and animals, since the bacterial resistance to antibiotics has become a serious medical problem (Lorch, 1999).

T4 phage genome

In general, phage genomes share many properties with those of other viruses. They are relatively small (usually about 2–40 kbp (DNA) or kb (RNA)), contain very restricted uncoding nucleic acid regions and minimum of or no auxiliary compounds (e.g. histones). Phage or viral genomes are usually organized in one nucleic acid molecule.

T4 is a giant among phages, its genome contains 1.6×10^5 bp compared with 3×10^6 bp for *E. coli* and 2×10^7 bp for yeast. T4 phage contains a single circular DNA organized in one chromosome. The analysis of the genome sequence allowed to predict about 400 potential ORFs. More than 160 of them have been already characterized as functional genes (Kutter *et al.*, 1994).

Mutation is an inheritable change in DNA that influences the structure and/or function of the protein product encoded by a mutated gene. There are two general types of mutations: point mutations and chromosomal mutations (Lewin, 1994).

Point mutations, i.e. substitutions, deletions or insertions affect very small sections of genomes. These small changes

occur in the T4 phage genome very often. Chromosomal mutations are changes that are frequently observed in higher organisms. They affect large parts of DNA. Peculiarities of phage genome reduce the variety of chromosome mutations in phage DNA. Unless there is a compensating deletion or a mutation that increases the amount of DNA that can be packed, no duplication or insertion can be longer than the length of terminal redundancy, roughly 3.3 kbp. This is a requirement for DNA replication. In practice, reducing terminal redundancy by even 1.8 kbp may be mildly harmful, while a 2.5 kbp deletion of non-critical DNA appears to be innocuous. It is important that large changes in DNA cannot occupy vital genes, because the genome is haploid and no complementation occurs (Drake and Ripley, 1994). The T4-even bacteriophages family is very numerous and many T4 phage mutants have been described (Ackermann and Krisch, 1997). A genomic mutation can be induced spontaneously or by many different agents.

Spontaneous mutations in T4 phage

The spontaneous mutagenesis is a process that leads to mutations, usually of point type, which are generated by base substitution without participation of external physical and chemical factors or of genetic manipulations. Spontaneous mutations are a result of errors in DNA replication. The spontaneous mutation rate is related to the rates of insertion and removal of nucleotides during the synthesis of DNA (Muzyczka *et al.*, 1972). Its frequency in T4 phage is about 10^{-7} per bp in one replication cycle (Stryer, 1999). At some sites of T4 phage genome the mutation frequency is higher than at others, and these are called "hot spots".

Studies on the biochemical basis of spontaneous mutations are possible using mutator and antimutator T4 phage strains. Mutators are mutant phage strains with higher mutation rates than that of wild-type strains. They are easy to isolate and to detect because of their specific phenotype, which is extremely important for the experimental work with phages. This kind of mutants occurs frequently in the environment. Antimutators are mutant strains with lower mutation rates than that of wild-type strains. Antimutators decrease the efficiency of error-producing systems and, at the same time, increase the efficiency of the normal mutation-prevention systems (Schaaper, 1998).

T4 phage antimutators have been discovered among temperature-sensitive gene 43 mutants. They are highly specific because they reduce mutation rates of certain type only, usually AT→GC (Drake, 1966, 1988). Later studies have shown that small increases in the transversion frequency were observed (Ripley, 1975). Ripley and Shoemaker (1983) have also disclosed an interesting dichotomy in the case of frameshift mutations. This kind

of mutations changing the reading frame was reduced in antimutator phage strains.

Antimutators have been found in several other phages since their discovery in T4 phage (Drake *et al.*, 1969; Rehakrantz, 1988). Historically, they have generally received less attention than their counterpart, mutators. The main reason for this is that antimutator strains are more difficult to detect and isolate.

Defects in genes that encode proteins involved in DNA metabolism often result in mutator or antimutator phenotypes. Spontaneous mutations in the T4 phage DNA polymerase gene perturb its exonuclease-to-polymerase ratio. These mutations may produce either mutators or antimutators, depending on which kind of T4 phage polymerase activity is mutated. It has been reported that a missense mutation in the structural gene for the T4 phage-specific DNA polymerase (gene 43) produced an altered enzyme with a reduced base selectivity. A non-complementary base could be inserted more frequently into the DNA of these mutants during replication, leading to an increased mutation rate (Muzyczka *et al.*, 1972).

Bebenek with coworkers (Bebenek *et al.*, 1999) have noticed that T4 phage RNase H null mutations affect spontaneous mutation, namely reduce burst size, increase sensitivity to DNA damage, and increase the frequency of T4 acriflavin resistance mutations, increase the sensitivity to lethal effects of both UV irradiation and the topoisomerase inhibitor. The T4 phage RNase H null mutations might display a mutator activity, and this activity might include a bias toward duplication.

Mutagenesis induced by physical and chemical mutagens

A mutation can be induced by many physical and chemical mutagens. The earliest studies on the molecular basis of mutation caused by such mutagenes have been conducted using T4 and other T-even phages (Drake, 1963, 1966 a,b). The results of these studies were very often applicable to higher organisms due to similar mechanisms of mutagenesis.

UV irradiation is one of the basic physical mutagens that induce many different photochemical lesions in DNA, but mainly pyrimidine-pyrimidine cyclobutane dimers and pyrimidine (6-4) pyrimidone dimers. Both of these contribute to lethal effects and mutagenesis in cellular systems (Drake and Ripley, 1994). T4 phage is inactivated by UV light at a wavelength of 253.7 nm (Freese and Freese, 1961). The analysis of UV mutagenesis is very much complicated by two factors: (i) the putative target molecule-DNA resides within a complex and chemically highly reactive environment, and (ii) the variety of repair processes that have taken place before the UV damage are expressed

as heritable lesions. Photoreactivation is the main repair process. Mutation in a single gene *v* which controls the extent of photoreactivation causes increased UV sensitivity in T4 phage (Harm, 1963).

Another physical mutagen is heat. It easily allows the induction of substitution mutation in T4 phage. At 37°C the heat-induced mutation rate is about 4×10^{-9} per GC base pair per hour (Kricker and Drake, 1990). This is a very high rate of heat mutagenesis when extrapolated from T4 phage to higher organisms (Baltz *et al.*, 1976). Heat mutagenesis in T4 generates exclusively a base pair substitution at GC. Neither base pair substitution at AT nor frameshift mutations are induced by heat (Bingham *et al.*, 1976).

UV irradiation and heat may represent examples of a class of mutagens that are more important in evolution than are ordinary errors in DNA replication (Baltz *et al.*, 1976). Many chemical substances belong to the group of very well-known mutagens, e.g. base analogs, hydroxylamine, ethyl methanesulfonate, methyl methanesulfonate, acridine, and nitrous acid.

The base analogs 2-aminopurine, 2,6-diaminopurine and 5-bromouracil produce transition mutations (AT \leftrightarrow GC) different from those arising spontaneously. In T4 phage, 2-aminopurine preferentially induces the GC \rightarrow AT transition, while 5-bromouracil preferentially induces the AT \rightarrow GC transition. Very interesting is the fact that 2-aminopurine-induced mutants are readily reversible by 5-bromouracil and vice versa (Freese, 1959a,b; Litmann and Pardee, 1956).

Hydroxylamine is a highly effective mutagen that modifies cytosine far faster than any other base and causes the unidirectional transition GC \rightarrow AT (Freese *et al.*, 1969a,b; Strauss, 1961).

DNA-alkylating agents such as ethyl methanesulfonate and methyl methanesulfonate were among the first compounds found to be mutagenic for T-even phages. Ethyl methanesulfonate induces the GC \rightarrow AT transition strongly while the AT \rightarrow GC transition weakly. This occurs in two different ways, (i) by mispairing alkylated bases or (ii) by alkylation-induced depurination followed by a non-template misinsertion (Lawley and Martin, 1975; Strauss, 1961). Methyl methanesulfonate also causes the GC \rightarrow AT transition by directed miscoding (Drake, 1988). Ethyl methanesulfonate and methyl methanesulfonate are effective when applied to free phage particles, however there is a lot of alkylating agents that are mutagenic only for intracellular T4 phage (e.g. *N*-methyl-*N*-nitro-*N*-nitrosoguanidine or *N*-methyl-*N*-nitrosourea) (Drake and Ripley, 1994).

Acridine and its derivatives, such as proflavine (3,6-diaminoacridine), are classical intercalating agents that induce frameshift mutations in T4 phage. A frameshift arises as a consequence of the misalignment of one DNA strand upon its complement in the course of DNA replication (Wang and Ripley, 1998). Such misalignment specifically predicts

duplication or deletion of a repeated DNA sequence. The sequences of acridine-induced frameshift in T4 phage are the longest runs of AT pairs. The specificity of proflavine in T4 phage is different from that reported in lambda and other phages or in *E. coli* plasmids, where frameshifts take place in repeated sequences of GC pairs. That distinction predicts organism-specific differences in DNA structures and enzymes involved in mutational mechanism (Ripley and Clark, 1986). Nitrous acid reacts rapidly and diversely with biological molecules containing extracyclic amino groups. The predominant reaction is oxidative deamination. Nitrous acid mutagenesis turns cytosine into uracil, 5-methylcytosine into thymine, adenine into hypoxanthine, and guanine into xantine (Stryer, 1999). This causes transitions, more strongly in the direction GC→AT (Freese and Freese, 1961). The rates of deamination and mutation induced by nitrous acid are similar in single-stranded and double-stranded DNA. The HNO₂ is effective either when applied to extracellular phages or to phage-infected cells (Baylor and Mahler, 1962).

Site-directed mutagenesis in T4 phage

Progress in genetic and molecular biology techniques gave possibility of manipulation genomes of viruses and other organisms. The practice of genetics no longer depends on isolating naturally occurring mutants. Site-directed mutagenesis is a technique used in genetic engineering for introducing mutations into the selected genes.

Particular changes in the nucleotide sequence of a gene allow to modify precisely the amino acid sequence in the expressed protein product of the gene to investigate how such a change affects the protein's structure and function. It is also possible to change the DNA sequences that function as genetic regulatory or "control" elements (Darnell *et al.*, 1986; Martin and Hine, 2000). Polymerase chain reaction and artificial oligonucleotides of defined sequences may be used to introduce changes selectively and specifically by site-directed mutagenesis (Griffin and Griffin, 1995). Such manipulations of phages can influence their affinity to bacteria and can be a practical method of enriching phage collections, which is a very important prerequisite of the present day phage therapy.

The complete T4 phage genome has been sequenced (Ackermann and Krisch, 1997; Kutter *et al.*, 1994) which is an important step for the site-directed mutagenesis. The insertion/substitution system devised by Selick and co-workers allows a variety of genetic manipulations of T4 phage, most importantly the transmission of *in vitro*-generated mutations from a plasmid into the phage genome. The vector pBSPL0+ used in this method is a 2638 bp plasmid containing an origin of DNA replication and ampicillin resistance determinant, both derived from plasmid

pBR322 (Sutcliffe, 1978, 1979; Watson, 1988), a promoter from T4 phage gene 23 (*supF*), and a polylinker with eight unique restriction enzyme recognition sites.

A "target" sequence from T4 phage DNA is cloned into this vector and then mutated by standard DNA recombinant techniques. What is important is that the constructed mutant can be either analyzed directly for a possible mutant phenotype or transferred to non-selective growth conditions (Mattson *et al.*, 1983; Selick *et al.*, 1988). The insertion/deletion system provides a general method that can be applied to any segment of the T4 phage genome.

The site-directed mutagenesis has helped to understand the functions and structures of some important gene products. The amino acids localized outside of an active site of enzyme (in an enzyme core) are much more tolerant for changes in genetic code like insertions and substitutions.

The site-directed mutagenesis has been induced in many genes of T4 phage such as lysozyme, DNA polymerase, endonuclease V, regA protein and others. T4 phage lysozyme is a monomeric protein of 164 amino acids that hydrolyses peptidoglycan. Its role in the lytic cycle of phage is to start the extracellular phase by liberating phage particles from the metabolically inert remains of the bacterial host. Mutant strains of T4 phage lacking lysozyme function are defective in lysis but can be rescued by externally added lysozyme in the presence of agents that destabilize the outer membrane of the host. Lysozyme has played a significant role in securing the present understanding of the genetic code in viruses and also served as a useful model system in studies of protein folding/unfolding (Poteete and Hardy, 1994). A systematic site-directed mutagenesis of T4 phage lysozyme has been carried out. Amber mutations were introduced into each codon except the first one and the resulting amber mutants were analyzed. This technique permitted assessment of the effect of 12 or 13 single amino acid substitutions at each position in the protein, for a total of 2015 variants (Rennel *et al.*, 1991). This experiment indicated that T4 lysozyme tolerated a single amino acid substitution in 89 out of 163 positions (55%). Especially sensitive sites included the key catalytic amino acid and a few others that may have a critical structure role.

Experiments with T4 phage lysozyme illustrate the importance of relatively unbiased genetic approaches to structure-function studies. The use of site-directed mutagenesis to test many hypotheses derived from the investigation of protein structure is unquestionably powerful and fruitful.

T4 phage DNA polymerase plays a central role in replication process since it catalyzes both template-directed 5'-3' synthesis of DNA and excision of nucleotides from 3'-termini, an activity crucial for maintaining the fidelity of DNA replication (Clayton, 1999; Kunkel, 1988; Reddy *et al.*, 1992; Satar *et al.*, 1996). It was found that a single mutation decreases the enzyme processivity as a polymerase and increases its processivity as a 3'-5' exonuclease

(Spacciapoli and Nossal, 1994). Site-directed mutagenesis in gene 43 encoding T4 phage DNA polymerase is useful in understanding the role and interactions among the proteins involved in the synthesis of the new DNA (Kunkel *et al.*, 1984; Karam and Konigsberg, 2000).

The T4 phage regA protein is a translational repressor that regulates the synthesis of more than 12 proteins. Amino acid substitutions were introduced at Phe-106 to evaluate its role in nucleic acid binding. Two deletion mutants (1-94, 1-109) were cloned and purified. The binding affinities of these mutants to polyuracil were reduced. All the studies so far made indicate that the last 13 amino acids in the regA protein contribute significantly to RNA binding (O'Maley *et al.*, 1995). It was the site-directed mutagenesis that helped to disclose the function of regA protein.

T4 endonuclease V is an enzyme which has both N-glycosylase and apyrimidinic/apurinic lyase activities (Schrok and Lloyd, 1991). The site-directed mutagenesis of the N-terminus of endonuclease V was used to investigate the important parameters involved in the cleavage mechanisms. Mutants with three substitutions were prepared: Thr-2 to Ser-2, Val-2, and Pro-2. The results indicated that a critical parameter for the functionality of the enzyme was the relative distance between the primary α -NH₂ group at the active site of the enzyme and the structures responsible for DNA binding and pyrimidine dimer recognition (Schrok and Lloyd, 1993).

The site-directed mutagenesis is a method that allows to construct large collections of specific mutants. It is very important for studying the three-dimensional (3D) structure of proteins because comparison of the wild type proteins with mutants can give a detailed information about the features of the recognition determinants and "hot spots" of phage-bacterium interaction. Most of known 3D structures of T4 phage polypeptides include enzymatic proteins. Determination of the 3D structure of the structural proteins responsible for the phage-bacterium recognition would be of invaluable help in rational design of phages with altered specificity. There are only a few well described 3D structures of T4 phage proteins in databases (<http://www.rcsb.org/pdb>, <http://www.biochem.ucl.ac.uk/bsm/pdbsum>), e.g. lysozyme, endonucleases V and VII, β -glucosylotransferase, glutaredoxin, hydroxymethylase, gp11, gp12, gp27, gp31, gp32, and gp45.

The site-directed mutagenesis is also used for studying genomes and proteins of many pathogenic viruses as poliovirus (Barton and Flanagan, 1993; Diamond and Kirkegaard, 1994; Jablonski and Morrow, 1995; Teterina *et al.*, 1995; Walker *et al.*, 1995), herpes simplex (Wu *et al.*, 2002), influenza virus (Sakai *et al.*, 2002), hepatitis C virus (Ingallinella *et al.*, 2002), rabies virus (Wu *et al.*, 2002) and many others. This knowledge is extremely important for medical treatment and prophylactic vaccination.

Obviously also many higher organisms have been tested using site-directed mutagenesis techniques. Many enzymes

of bacteria (Pascarella *et al.*, 1998; Milla *et al.*, 2002; Orlik *et al.*, 2002), protozoa (Hannaert *et al.*, 2002), plants (Fairclough *et al.*, 1998) and animals (Church *et al.*, 2002; Huang and Colman RF, 2002; Peelman *et al.*, 1998; Pei *et al.*, 2002) are being nowadays investigated by site-directed mutagenesis.

Conclusions

Phages are invaluable models for understanding many biochemical and medical issues. Because of their potential usefulness in therapy of bacterial infections, it is very important to examine accurately physiology and life cycle of phages. The site-directed mutagenesis is a method that enables one to introduce mutations which can influence phage affinity to bacteria and it can be a practical technique for enriching phage collections and widening phage specificity for evolved bacterial strains that are at present insensitive to phage therapy. The site-directed mutagenesis appears to be the most applicable method because it is precise and feasible to design (Tétart *et al.*, 1996).

The stage in which T4 phage binds to receptors on the bacterial surface and the molecular basis of this process seems to be an interesting subject for further investigation. It can be expected that further progress in intervention into phage genome could lead to the development of more efficient means of anti-bacterial therapy.

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