Translational, Autogenous Regulation of Gene 32 Expression during Bacteriophage T4 Infection

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Functional half-life measurements of the bacteriophage T4 gene 32 messenger RNA indicate that this mRNA is extremely stable. Regulation of gene 32 expression at the transcriptional level cannot account for the rapidity with which P32 synthesis can be repressed. Furthermore, derepression of P32 synthesis occurs in the presence of rifampicin, a drug which inhibits transcriptional initiation. In addition, T4-infected cultures in which P32 expression is repressed possess almost as much gene 32 mRNA as derepressed cultures. We conclude that expression of the T4 gene 32 protein is regulated at the level of translation.

During infection of Escherichia coli by the bacteriophage T4 a large number of virus-encoded proteins are synthesized (1, 2). Among these proteins is a DNA-binding protein (the gene 32 protein, P32) which was first characterized by Alberts and his colleagues (3, 4). Gene 32 expression is autogenously regulated; infections by gene 32 mutants yield abnormally large quantities of the altered polypeptide (5, 6). Regulation of gene 32 expression is normally regulated by the quantities of intracellular single-stranded DNA (5), a known substrate for P32 binding in vitro (3, 4).

Since P32 can bind to RNA as well as to single-stranded DNA (7–10), we asked whether regulation of gene 32 expression occurs at the translational or transcriptional level. Our results show that autoregulation of gene 32 expression is translational; to our knowledge, gene 32 expression is the first example in E. coli of translational control in which the regulation could have been transcriptional.

MATERIALS AND METHODS

Bacteriophage Strains—The sources of bacteriophage strains are indicated in the preceding paper (5). In the text and figure legends we have designated the genotype for specific mutations as X for amber wild type genotype is designated X +.

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† The abbreviations used are: P32, protein encoded by wild type gene 32; "P32," protein (or protein fragment) encoded by gene 32 mutants; SDS, sodium dodecyl sulfate; am, amber mutation; to, temperature sensitive mutation.

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FIG. 1. Functional half-life of gene 32 mRNA. Bacteria (AS19) were infected with amHL618-amE2059-deletion r638 (32' 44-rIIB') phage at a multiplicity of eight. Five minutes after infection, rifampicin was added to the culture, and at the times indicated 1-ml aliquots were pulse-labeled with 32P-amino-acids (1 μCi/ml). The data are expressed as a per cent of the values obtained for the radioactive pulse at 11 min postinfection. The symbols are: ○, 32P32; ●, total 32P-amino-acid incorporation; □, P43; ■, PIIA; △, P46; ▲, P52. We have included a reference line (---) to indicate the slope for a 3-min half-life.

Measurements of DNA Synthesis—DNA synthesis was measured in 1-ml aliquots of infected cells during 1-min pulses with [3H]thymidine at 1 μCi/ml (2). The radioactive thymidine was from Amersham ([methyl-3H]thymidine, TRK 120, about 15 Ci/mmol).

RESULTS

I. Gene 32 mRNA Half-life—Since rifampicin is a known inhibitor of all transcriptional initiations which occur during T4 infection (2, 16, 17), that antibiotic may be used to determine functional messenger half-lives (18, 19). The functional half-life of the gene 32 mRNA was determined during infection by an amber mutant in gene 32 (Fig. 1). Whereas the average functional decay of T4 mRNA, as measured by total protein synthesis, occurs with a half-life of 6% min (in agreement with the published data of others (19)), most specific T4 messengers are less stable than the average. On the other hand, mRNA encoding the gene 32 amber fragment is completely stable. Due to possible effects of rifampicin addition (from 5 to 30 min postinfection) did not affect the data. When measured under repressed conditions, that is, in a 45-32' infection (5), the apparent half-life of gene 32 messenger RNA is shorter than its half-life when measured under derepressed conditions (that is, for example, in a 45-32' infection (5)); however, the gene 32 mRNA under any conditions is more stable than any other T4 mRNA. The data from more than 10 determinations yield a half-life of greater than 30 min for gene 32 mRNA under derepressed conditions, and a half-life of more than 15 min for the same mRNA under repressed conditions. The P-fold difference in gene 32 mRNA stability cannot account for the vastly different rates of P32 synthesis observed (5); for the relative half-lives calculated for repressed and derepressed gene 32 mRNA it would take 1 h to generate a 4-fold difference in the relative rates of P32 synthesis.

The stability of the gene 32 mRNA (under any condition) suggests that efficient transcriptional regulation cannot occur; rapid transcriptional repression of a gene which produces an extremely stable mRNA cannot quickly alter the rate of expression of that gene. Therefore, experiments (below) have been performed which explicitly test the hypothesis that the regulation of gene 32 expression is translational.

II. Denaturation of Thermolabile Repressor and Derepression in Presence of Rifampicin—P32 is its own repressor. Using a temperature-sensitive gene 32 mutation and an appropriate...
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Fig. 3. Derepression of P32 synthesis after rifampicin addition. Bacteria (AS19) were infected at 39°C with amN134-amBL292-amB263 (33-55-43°), amN134-amBL292-tsP36 (33-55-43°), or amN134-amBL292 (33-55 °) phage at a multiplicity of 10. Rifampicin was added to each culture at 8 min postinfection (RIF); the cultures were transferred to 30°C at 9 min. Aliquots (1 ml) of the cultures were pulse-labeled for 1 min at various times either with [3H]thymidine (1 μCi/ml) (Fig. 3A) or with [14C]-amino-acids (1 μCi/ml) (Fig. 3B). The data have been corrected for the rates of isotope incorporation at the two temperatures (see “Materials and Methods”). The symbols are: ○, 33-55-43°; □, 33-55-43°; △, 33-55°.

Accordingly, two triple mutants of T4 were compared: 33-55-43°, in which the gene 43 mutation is an amber, and 33-55-43° in which the gene 43 mutation (tsP36) renders the gene 43 protein thermolabile (gene 43 encodes the T4 DNA polymerase (25)). At high temperature the two phages are equivalent; neither synthesizes DNA, and as a result, gene 32 expression is low. However, if infections are initiated at high temperature and then shifted to low temperature, this thermolabile DNA polymerase renatures (26) and allows DNA replication (Fig. 3A). Thus, one may ask if gene 32 derepression in response to newly generated single-stranded DNA can occur in the absence of transcriptional initiations.

The rate of gene 32 expression in tsP7 infected cultures is about 7 times higher at 42°C than at 20°C; the rate of gene 32 expression in tsP7 at 42°C is equivalent to the fully derepressed rate observed for the gene 32 amber mutant. The synthesis of the gene 32 amber fragment used as a control is not sensitive to temperature shift.

“P32” overproduction is not severe in tsP7 infected cells at 20°C; thus this culture has the capacity for substantial derepression which can be uncovered by temperature shift. Approximately 20 min at 42°C are required for the tsP7 infected culture to fully derepress gene 32 expression; this particular ts loses the capacity to replicate DNA after a much shorter interval at 42°C (21, 22).

III. Derepression of P32 Synthesis after Rifampicin Addition—Derepression of wild type gene 32 protein occurs if single-stranded DNA accumulates in cultures infected with phage of the appropriate genotype (5). The T4 double mutant 33-55° (12, 23, 24) yields extremely high levels of gene 32 expression; that high level expression is dependent on DNA replication (5).

1 Unpublished experiments from this laboratory.
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Data have been corrected for the rates of isotope incorporation at the time of labeling with 5-amino acids (1 μCi/ml) for 1 min at various times. The bacteria (AS19) were infected at 30°C with amN134-amBL292 (33-55-) or amN134-amBL292-20766 (33-55-43t) minus rifampicin; A, 33-55-, plus rifampicin; O, 33-55-43ts, two temperatures (see "Materials and Methods"). The symbols are: O, 33-55-, infected cells; A, 33-55-, plus rifampicin; Δ, 33-55-43ts, minus rifampicin.

Gene 32 protein synthesis drops rapidly in the 33-55-43ts infected cells (Fig. 4). The important additional result is that rifampicin addition prior to the temperature shift has no effect on P32 expression (Fig. 4). Therefore, the maintenance of derepressed gene 32 mRNA (see I, above); thus, the slow loss of intracellular gene 32 mRNA remains high in the 33-55- infected cells (Fig. 4). The importance of this result is that rifampicin addition prior to the temperature shift has no effect on P32 expression (Fig. 4). Therefore, the maintenance of derepressed gene 32 messenger in the 33-55- infection does not result from continued, rapid transcriptional imitations.

V. Intracellular quantities of gene 32 mRNA—Since the gene 32 mRNA is stable (see I, above), repression of gene 32 expression should not be accompanied by an equivalent loss of translatable gene 32 mRNA. Intracellular gene 32 messenger RNA concentrations have been measured using cell-free translation of that messenger as an assay (14). The gene 32 mRNA (present in crude RNA extracted from appropriate T4-infected cells) is an efficient messenger for the gene 32 protein (Fig. 5). Gene 32 messenger isolated from cells infected with a gene 32 amber mutation directs the synthesis of the correct amber fragment; efficient suppression of that amber site is achieved in vitro with Suv+ tRNA (Fig. 5) or Suv+ tRNA. The assays described below have been carried out under limiting mRNA input; these conditions allow quantification of the relative abundance of gene 32 mRNA in a given crude RNA preparation (14).

The autoradiogram (Fig. 5) contains more than a demonstration that gene 32 mRNA can be assayed in vitro. In addition, a comparison is offered between gene 32 expression in vivo and the relative quantities of intracellular gene 32 mRNA. Cultures were infected with either 33-55- or 46- phage (27); at 30 min postinfection the 46- infected culture yields P32 at 2.5% the rate observed in the 33-55 infected culture (Fig. 5, F and G) (see also Table I, Table IV, and Fig. 8 of the preceding paper (5)). By contrast, the RNA extracted from the 46- infected cells directs the synthesis of P32 at nearly 40% the level of RNA extracted from 33-55- infected cells (Fig. 5, D and E).

Intracellular gene 32 mRNA levels also have been measured as a function of time and temperature in parallel infections by either 33-55- or 33-55-43t- phages; the conditions used were similar to those shown in Fig. 4. Infections were initiated at 30°C; 20 min after infection the cultures were shifted to 42°C. At various times after infection, two separate measurements were made: first, the level of P32 synthesis in vivo, and, second, the level of P32 synthesis in vitro in response to RNA extracted from the infected cells. For both the in vivo and in vitro results, the data are plotted as the ratio obtained for 33-55-43t- to 33-55- infections (Fig. 6). Whereas the 33-55-43t- infected cells undergo a 10-fold reduction in the relative rate of P32 synthesis following temperature shift to 42°C, only a small reduction in the intracellular level of gene 32 messenger RNA is observed. Although the data for translation in vitro scatter considerably, the same result has been obtained in three separate experiments. The level of intracellular gene 32 message, as assayed by cell-free translation, does not fall to a level corresponding to the diminution of P32 synthesis in vivo.

Similarly, the levels of gene 32 mRNA have been measured in cultures infected by either 33-55- or 33-55-46- phages. Each culture makes DNA at wild type rates (5, 28); however, at about 10 min postinfection the 33-55-46- infected cells begin to synthesize less P32 than the 33-55- infected cells; at 25 min postinfection the relative amount of P32 expression in the two infected cultures differs by approximately 5-fold (Fig. 7). The amount of gene 32 mRNA in 33-55-46- infected cells does not fall below half that of the 33-55- infected cells (Fig. 7). Once again, the intracellular gene 32 mRNA remains relatively high in cells which are repressed for gene 32 expression.

Repressed gene 32 mRNA is somewhat less stable than derepressed gene 32 mRNA (see I, above); thus, the slow loss of mRNA with following repression (Figs. 6 and 7) and the concomitant accumulation of perhaps 3-fold more gene 32 mRNA in fully derepressed versus fully repressed conditions (Fig. 5, D and E) are not surprising. However, repressed cultures contain severalfold more intracellular gene 32 mRNA than predicted by transcriptional regulation models.

Repressed mRNA can be derepressed (that is, translated) in the absence of transcription. A comparison has been made between cells infected with 33-55- and 33-55-43t- phage during a double temperature shift regime. Infections were started at 30°C, rifampicin was added, and the cultures were shifted to 42°C. In the 33-55-43t- infected cells, DNA synthesis stops abruptly and P32 expression drops within 4 min to 20% that of the 33-55- control infection (Fig. 8). The cultures were then shifted back to 30°C, at the time of the return to 30°C the level of intracellular gene 32 mRNA in the 33-55-43t- infected cells should have been (Fig. 6) about 70% that of the control. In striking support of translational regulation, after resumption of DNA synthesis in the 33-55-43t- infection, gene 32 expression climbs to 70% of the P32 expression of the control infection (Fig. 8).

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Fig. 5. Translation of the gene 32 mRNA in vitro. Cell-free protein synthesis (autoradiograms A to E) was performed in 0.05-ml reaction mixtures as described previously (16). Sample A shows the pattern of protein synthesis for no added template. Reactions B and C were primed by 1.3 A_{300} units of total RNA extracted 40 min postinfection from bacteria infected by amB271 (46') and amB271 (46') phage, respectively. Reactions B to E were primed by 1.3 A_{300} units of total RNA extracted 31 min postinfection from bacteria infected by amB271 (46') and amN82-deletion r638-amHL618 (45-44 rII-32') phage; reaction C contained, in addition, 0.005 A_{300} unit of purified (1142 pmol of serine aminoacylated/A_{300} unit)

**DISCUSSION**

**P32 Probably Represses Translation Directly**—A rather simple notion may be utilized to explain the data presented here and in the preceding paper (5). The gene 32 protein has two quite different biochemical fates: P32 binds either to regions of single-stranded DNA, thereby promoting DNA replication, repair, or recombination, or, if no single-stranded DNA is available, P32 binds to its own messenger RNA and represses its own translation. A logical case has been made that overproduction of P32 results from the appearance of single-stranded DNA (5) and, conversely, that repression of P32 translation is brought on by the absence of single-stranded DNA; however, the notion that P32 directly represses its own synthesis is merely the least complicated and most appealing model consistent with the data. No mutants in essential T4 genes (other than gene 32 itself) alter P32 expression in a manner consistent with an activator or repressor function. Only mutants in gene 32 give increased expression of gene 32 independent of the state of the intracellular DNA (5). In addition, the gene 32 protein harbors a strong binding site for single-stranded DNA and RNA (3, 4, 7, 8). Thus, repression could utilize the nonspecific binding site and might be, thereby, “economical.” During infections by all temperature-sensitive gene 32 mutants at permissive temperatures (where DNA replication proceeds at wild type rates), the altered P32 is always somewhat overproduced. Such low temperature conditions are fully permissive for all parameters of phage growth. The amount of functional gene 32 protein in such infections must be equivalent to the wild type level (5). Therefore, the overproduction of thermolabile P32 species at permissive temperatures most likely results directly from the diminished repressor function of these temperature-sensitive proteins. Thus, we argue that P32 is its own repressor, and, as documented above, the molecular target for repression is the gene 32 mRNA.

**Observations Consistent with Translational Model**—RNAs synthesized after T4 infection have been examined on polyacrylamide gels; there is a prominent RNA, about 1200 nucleotides long, which has been tentatively identified as the gene 32 mRNA on the basis of cell-free translation experiments. Infected cells which make little P32 in vivo have
slightly reduced levels of putative gene 32 mRNA, but that RNA remains a major band; the reduction in intensity presumably is related to the shorter half-life of repressed mRNA (see above). This RNA is the most prominent large RNA at late times in a 33-55 mRNA and is completely stable after rifampicin addition. Lastly, this RNA is observed only if the labeling period is long; short pulses of [14C]uridine are never preferentially incorporated into this RNA. Further support of the translational model is provided by the observation that translation of gene 32 mRNA is efficiently and specifically repressed in vitro by added P32. These experiments will be reported subsequently.

Molecular Aspects of P32 Repression of Gene 32 mRNA—The translational repression of gene 32 messenger RNA could be a function of specific sequence recognition by P32. Although gene 32 protein binds to single-stranded DNA and RNA without apparent sequence specificity (3, 4, 7-10), one might argue that the translational repression of gene 32 messenger RNA involves specific sequence recognition. Such a model would be merely derivative of the classic operon models for transcription (29); the repressor binding site (in the vicinity of the initiation site for translation) would be equivalent to an operator (30). In fact, repression of the translation of the R17 synthetase cistron by the R17 coat protein is similar to classic transcriptional repression (31, 32).

However, the gene 32 protein does not show sequence recognition in vitro, whether for DNA or RNA binding (3, 4, 7, 8); can specific repression emerge without sequence recognition? The answer is "perhaps" if one is willing to imagine that gene 32 mRNA contains unusual structural elements in the vicinity of the ribosome binding site. We offer below a speculative model that provides a gene 32 messenger RNA which is simultaneously (a) stable, (b) capable of extremely efficient (derepressed) translational initiations, (c) repressible by the gene 32 protein (Fig. 9). Other T4 mRNAs must be constructed in such a manner as to preclude long half-lives and P32-mediated repression.

The details of this model are as follows: the gene 32 mRNA initiation region contains an unusually long region devoid of base pairs (33); the decanucleotide sequence UAAGGAGUUG (34) is positioned seven bases distal to the initiator AUG to ensure strong homology with the 3' end of 16 S RNA (35); the region is constructed so that either 1 ribosome or 5 P32 molecules can just cover the site; no base pairing is allowed within this region, unlike random RNA (33) and presumably unlike other T4 mRNA initiation regions. The structure is
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Fig. 8. Repression and derepression of P32 synthesis: repressed gene 32 mRNA can be translated after derepression. Bacteria (AS19) were infected at 30° with umN134-amBL292 (33-55°) or umN134-amBL292-tsP36 (33-55-43°) phage at a multiplicity of 10. At 18.5 min postinfection rifampicin was added (RIF), and 1.5 min later the cultures were transferred to 42°. At 24 min postinfection the cultures were returned to 30°. Aliquots were pulse-labeled with 14C-amino-acids (1 μCi/ml) or with [3H]thymidine (1 μCi/ml) for 1 min at various times during the experiment. The data have been corrected for the rates of isotope incorporation at the two temperatures (see “Materials and Methods”). In the upper panel the rates of [3H]thymidine incorporation are plotted as the ratio [3H]thymidineß3-3s3/[3H]thymidineßß. In the lower panel the rates of P32 synthesis in the two cultures are shown: O, cells infected by 33-55° phage; O, cells infected by 33-55-43° phage. In the middle panel the data from the bottom panel are replotted as the ratio P32ß3-3ß/P32ßß.

Fig. 9. A model for autogenous, translational regulation of gene 32 expression. The 5' end of the gene 32 mRNA is shown containing a hypothetical decanucleotide sequence which complements the 3'-OH end of Escherichia coli 16 S RNA (35). This mRNA has two primary biochemical fates: repression by P32 or initiation of protein synthesis by the E. coli protein synthesis apparatus. The special features of the 5' end of this mRNA are discussed in the text.

quite analogous to the ribosomal binding site of the A cistron of the bacteriophage R17 (34), except that the secondary structure of gene 32 mRNA surrounding the initiator region does not interfere with ribosome access. By suitable adjustment of binding constants of P32 toward single-stranded DNA and RNA, rapid derepression by gapped DNA (through competition for P32 with the gene 32 mRNA initiation region) may be facilitated (5). Random nucleotide sequences the length of an initiation region (approximately 35 nucleotides) contain considerable secondary structure (33). Thus, in the absence of positive evolutionary pressure, other T4 messenger RNAs would not contain similarly constructed single-stranded sites in their initiation regions.

This model provides, through one unique structure and sequence homology with 16 S RNA, plausible explanations for several aspects of gene 32 regulation. Ribosomal attack of the derepressed mRNA must be extremely fast; we have calculated that the polypeptide elongation rate probably limits initiation on the derepressed gene 32 mRNA. The rapid and reversible repression of P32 synthesis (Fig. 8) suggests that the primary competitor for ribosomal attack is P32 itself; therefore, under derepressed or repressed conditions endonucleolytic attack is restricted by the ribosomes or repressor.

Furthermore, the model utilizes known molecular aspects of the gene 32 protein. The nonspecific binding site for single-stranded nucleic acids may be sufficient for repressor function; in fact, missense mutants in gene 32 are each deficient for DNA synthesis and repression. Cooperative binding of P32 to nucleic acids (3, 4), coupled with an initiation region lacking secondary structure, might enhance the selectivity of P32 binding to its own mRNA; the cooperativity also would provide more complete protection against endonuclease(s).

A last feature of the model relates to potential regulatory mutations. If the initiation region lacks internal base pairing, a single base change could not sufficiently increase the secondary structure to prevent P32 attack. Thus this model, unlike classic operon models, does not have the potential for mutations to constitutive gene 32 expression.

An entirely distinct model, in which the gene 32 protein attacks the ribosome and thereby alters translation, has not
been considered seriously; however, we must note that earlier workers probably identified P32 as a translational “initiation factor” (36).

Translational Regulation of P32 Expression Solves Difficult Problem.—The gene 32 protein must cover single-stranded DNA quickly in vivo or that DNA will be attacked by nucleases (5, 37); on the other hand, for a variety of reasons, gross overproduction of P32 relative to the number of single-stranded DNA binding sites, might be lethal (5). Thus, the P32 regulatory system provides rapid derepression and repression, since the risks of imprecise P32 regulation are large. Translational regulation is more rapid than transcriptional regulation (except in the case of a gene whose mRNA is infinitely unstable). Furthermore, the first events in derepression by single-stranded DNA probably involve capturing those P32 molecules which previously were bound to single-stranded molecules which previously were bound to single-stranded regions of mRNA. In addition to the initiation region of the gene 32 mRNA, these regions might include single-stranded sites within the coding portions of other T4 mRNAs. This derepression, in terms of the time needed to cover a gapped DNA, is more rapid than either transcription or translation. In a sense the mRNA pool (by virtue of its small quantity of non-base-paired regions) could hold a limited reservoir of P32, ready to instantaneously cover gapped DNA. The P32 reservoir could be replenished by subsequent translation. One must assume that P32 molecules which associate and dissociate rapidly from noninitiation regions of all cellular mRNA have no effect on the translation of those mRNAs; such a nonspecific reservoir is reminiscent of the postulated role of nonspecific DNA in the regulation of the lac operon (38).

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