Cell-Free Transcription and Translation of Isolated Restriction Fragments Localize Bacteriophage T5 Pre-Early Genes†

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Received 22 July 1985/Accepted 26 November 1985

In vitro transcription and translation of isolated restriction fragments containing all or part of the terminal redundancies of bacteriophage T5 localized virtually every pre-early gene to a small 6.3-kilobase *Bgl*I fragment. Among these genes were those encoding the A1 and A2 proteins, which are responsible for complete entry of the viral genome into its host, and the deoxynucleoside 5'-monophosphatase. A 3.9-kilobase *Bgl*I fragment containing the remainder of the pre-early region induced no proteins under these same conditions. Proteins induced by fragments including the right and left terminal redundancies were also compared and found to be identical. DNA immediately flanking the pre-early regions induced few proteins in vitro. Thus, this technique has allowed the overall gene organization of the pre-early region of T5 to be described.

The virus T5 is a medium-large coliphage whose mode of infection and consequent pattern of gene expression are of particular interest. During infection, viral DNA enters its host in two stages, with the first-injected small portion of the T5 genome (the first-step transfer DNA) encoding gene products which prepare the bacterium for subsequent entry of the remainder of the viral DNA (13). The gene products encoded by the first-step transfer DNA are designated pre-early proteins. The early proteins are induced soon after entry and expression of the rest of the viral genome, followed in turn by production of the late proteins. Thus, phage proteins are expressed in three phases (11, 12).

The mechanisms responsible for two-step genomic entry and sequential gene expression remain to be detailed, but pre-early proteins play a central role. These proteins include a factor which apparently modifies the host RNA polymerase (21), the products of the A1 and A2 genes which are required for complete entry of phage DNA into the cell (10), and a deoxynucleoside 5'-mono-phosphatase (22). However, the scarcity of common restriction enzyme sites in the first-step transfer DNA (16) and the presence of potentially toxic gene products hinder easy study of the pre-early region by cloning, though a 1.7-kilobase (kb) fragment from a mutant phage containing additional *Eco*RI sites in this region has been cloned by Brunel et al. (5). The relatively small number of phenotypically detectable mutations has also prevented complete mapping of pre-early genes.

A method offering insight into the location and organization of the genes in this region is isolation of restriction enzyme fragments containing pre-early DNA or some fraction of it, followed by use of these isolated fragments as templates for protein production in a cell-free coupled transcription-translation system. The labeled proteins can then be examined electrophoretically. An advantage of using isolated rather than cloned copies of a restriction fragment as templates for protein production is that the results are

uncomplicated by the presence of plasmid promoters and possible fusions between vector and viral proteins.

In this study, this technique was used to localize virtually all pre-early T5 proteins, including those of known function, to a relatively small 6.3-kb restriction fragment, thus defining the physical boundaries for the pre-early genes. In addition, parts of the T5 genome adjacent to pre-early regions were included in some fragments, allowing these neighboring portions of the genome to also be examined. A complicating feature of the T5 genome is that, as with other T-odd phages, its genome is terminally redundant (17). Furthermore, in T5, this terminal redundancy is very large and evidently includes the entire pre-early group of genes. Therefore the pre-early portion at one terminus has been assumed to be exactly repeated at the other end (17). When restriction fragments containing either end were compared in this work, the same polypeptides were induced, confirming the identity at the terminal repeats.

MATERIALS AND METHODS

Bacteria and bacteriophage. *Escherichia coli* F and F β and all T5 phage stocks were described previously (3). Growth of bacteria for isolation of phage DNA from infected cultures was done in nutrient broth supplemented with 1.0 mM CaCl₂. Growth of bacteria prior to UV irradiation and subsequent infection with T5 was done in maleate-glucose minimal medium, while the actual irradiation was carried out in maleate-glucose minimal starvation medium, which lacked glucose, and a source of sulfur (9).

Isolation of T5 restriction fragments. To obtain T5 DNA, *E. coli* was grown at 37°C to a concentration of about 5×10^8 cells per ml. Phage were added to give a multiplicity of infection of approximately 5, and the infected cultures were shaken at 37°C until lysis was evident about 2 to 3 h later. Chloroform was added to complete infected-cell lysis, and debris was removed by centrifugation for 10 min at 10,000 $\times$ g followed by virus sedimentation at 35,000 rpm in a Ti65 rotor spun by a Beckman L5 ultracentrifuge for 45 min. Phage were purified on a CsCl block gradient (4 ml, $\rho = 1.60$; 4 ml, $\rho = 1.40$; 3 ml, $\rho = 1.30$) in a SW41 rotor spun for 2 h. Phage were extensively dialyzed to remove CsCl and then

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FIG. 1. Diagram of T5 genome showing restriction sites for *BglI* (B) and *SalI* (S). Terminally redundant (TR) regions of the phage, at the extreme left and right ends of the genome, are included in the 13.2- and 15.2-kb *SalI* fragments, respectively. The numbers beneath the genome indicate (in kilobases) the total length of the phage genome and the sizes of the restriction fragments used for this study. The locations of the 10.2-kb terminal redundancies are also indicated. The positions of *BglI* and *SalI* sites are taken from Rhoades (16).

extracted with phenol saturated with TE buffer (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA) to obtain T5 DNA. Residual phenol was removed from the aqueous phase by extensive dialysis against 1 M NaCl followed by further dialysis against 10 mM NaCl-TE buffer. *BglI* and *SalI* were purchased from New England BioLabs, Inc., and used according to the specifications of the manufacturer.

After digestion, fragments of interest were isolated by electrophoresing the digest through an agarose gel (20 by 30 cm; SeaKem; FMC Corp., Marine Colloids Div.) until adequate resolution was obtained for the isolation of a given fragment away from neighboring bands. The fragment of interest was then run into Whatman DEAE-cellulose 81 paper (cleaned before use by soaking in 2.5 M NaCl followed by several changes of distilled water) inserted into the gel just ahead of the band; current was applied until the ethidium bromide-stained DNA of a given band had been run into the paper. Restriction fragments were extracted with 2 M NaCl-TE buffer, and ethidium bromide was removed by washing the extracted DNA with TE-saturated *n*-butanol. Sodium chloride and residual butanol were removed, and the DNA was concentrated with an Amicon Centricon 30-micro concentrator.

Cell-free protein production from isolated T5 restriction fragments. T5 restriction fragments were transcribed and translated in an Amersham procaryotic-DNA-directed translation system. This kit was used as prescribed by the manufacturer, with 2 μ l of 1,425-Ci/mmol, 16.8-mCi/ml [35 S]methionine (Amersham Corp.) per reaction. After the samples were prepared for electrophoresis as detailed before (2), sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the reaction products was performed essentially as described in the literature (8), except all gels were 10% in acrylamide. The gels were stained in 10% acetic acid and 30% methanol with Coomassie brilliant blue before being fixed overnight in the same solution without dye. Prior to autoradiography, the gels were treated with En³Hance (New England Nuclear Corp.), dried down onto Whatman 3MM paper, and then used to expose Kodak X-Omat AR X-ray film at -80°C with Du Pont Cronex Lighting-Plus intensifying screens. Exposure was for 6 to 12 h.

Preparation of ^{35}S -labeled extracts from UV-irradiated infected cells. *E. coli* F was irradiated with UV light to reduce host protein production during subsequent infection with T5, basically as described elsewhere (22), except that cells were grown in maleate-glucose minimal medium prior to irradiation. Viral protein products of infected cells were electro-

phoresed and autoradiographed as described above for cell-free reaction products.

RESULTS

Comparison of proteins made in vitro with proteins induced by bacteriophage T5 in vivo. Wild-type viral DNA was digested with the restriction endonuclease *SalI*, producing four fragments (Fig. 1). The 15.2- and 13.2-kb pieces, containing the right and left terminal redundancies, respectively, were isolated from agarose gels after being resolved from each other and from the two larger pieces. The 15.2- and 13.2-kb fragments were separated from each other by about 7 mm and from the large fragments by over 1.5 cm (data not shown). The separated right and left termini were then added to a cell-free transcription-translation system to determine what polypeptides they encoded.

To establish that the commercial cell extract responsible for transcription and translation of isolated T5 restriction fragments induced the same proteins as those made during infection of *E. coli* F, the products of the cell-free system were electrophoresed in parallel with proteins induced at various times postinfection from UV-irradiated *E. coli*. The results of such a comparison are shown in Fig. 2, with the 13.2-kb *SalI* fragment (left terminus) as a template in the cell-free system.

Generally, the cell-free and the pre-early in vivo protein patterns are quite similar. These data demonstrate that within 2 min after infection, T5 induces at least five major protein species represented by bands at 65, 40, 27, 16, and 14 kilodaltons (kDa). These are the expected sizes (11, 14). It is probable that the latter two bands result from a combination of closely spaced peptides unresolved by 10% polyacrylamide gels, because other workers, using higher-percentage gels, have observed several discrete bands between 16 and 12 kDa (14, 15). The prominent cell-free proteins match these 0- and 2-min postinfection in vivo peptides quite well, and even many minor bands are common to both patterns.

FIG. 2. Comparison of viral proteins induced cell-free by the isolated 13.2-kb *SalI* restriction fragment containing the T5 left terminal redundancy with proteins induced during infection of *E. coli* F with wild-type T5. The numbers immediately under the five in vivo lanes indicate the times postinfection (in minutes) the protein samples were taken from the infected culture. The numbers along the right edge of the gel show the sizes of protein standards in kilodaltons. The arrows indicate a putative early protein.

The pre-early nature of the cell-free proteins is proven by the immediate appearance postinfection of the corresponding band in the *in vivo* pattern (Fig. 2), followed by eventual fade-out and replacement with early proteins as the infection proceeds. Several characteristic pre-early functions can be observed among the complement of peptides induced by the 13.2-kb *SalI* fragment. Past research has identified the 65- and 16-kDa proteins as the products of the A1 and A2 genes, respectively (10, 11). When we used the 13.2-kb *SalI* fragment of T5 DNA, which carried a mutation in either the A1 or A2 gene, as a template in the cell extract, the appropriate band was missing (for the data for the A2 mutant, see Fig. 4). Past work also indicates that the 27-kDa pre-early protein is the deoxynucleoside 5'-monophosphatase (*dmp*) (15). Thus, there is good correspondence between 0- and 2-min postinfection *in vivo* and cell-free pre-early protein patterns, demonstrating that the *in vitro* system accurately transcribes and translates isolated phage DNA.

Comparison of cell-free proteins from right and left termini of T5. Figure 3 shows the proteins encoded by isolated left and right *SalI* fragments of T5. As expected, the two sets of proteins made *in vitro* are identical with one exception: a major band at about 29 kDa is synthesized in large amounts from the left end only. The overall similarity in protein patterns confirms that both ends of the viral DNA are transcriptionally similar *in vitro*. The exceptional band at approximately 30 kDa (indicated by arrows in Fig. 2 and 3) probably represents an early protein included along with pre-early peptides in the 13.2-kb *SalI* fragment, since this piece includes about 3 kb of DNA from the T5 early region. If so, the protein would be from the C region of the T5 genomic map, which directly adjoins the left pre-early region. A band moving just behind the 27-kDa pre-early protein appears *in vivo* by 5 min after infection (Fig. 2), when T5 makes the switch from pre-early to early proteins in infected cells, and this protein lines up with the protein synthesized only from the left end *in vitro*. Whatever assignment of function is made for this band, it is of interest that the 13.2-kb fragment contains this protein's presumably early promoter, which is read with the same facility as nearby pre-early promoters in the cell-free system.

Additionally, the 65- (A1), 27- (*dmp*), and 40-kDa bands all

FIG. 3. Comparison of protein patterns synthesized *in vitro* by right (R) and left (L) terminal redundancies contained within the 15.2- and 13.2-kb *SalI* fragments, respectively. The numbers along the right edge of the gel show the sizes of protein standards in kilodaltons. The arrow marks a putative early protein.

FIG. 4. Localization of T5 pre-early genes by comparison of proteins synthesized *in vitro* from the 6.3-kb *BglI* restriction fragment from the left end (L) with proteins induced by the 3.9-kb *BglI* fragment from the right end (R), as shown in the two center lanes. Bracketing these center lanes are the proteins induced by right (R) and left (L) *SalI* fragments from either wild-type T5 or phage mutant in the A2 gene. The numbers along the right edge of the gel indicate the sizes of protein standards in kilodaltons.

have comparable intensities within a lane, indicating that similar amounts of all three proteins are made in the cell-free system (Fig. 3). Incomplete resolution of proteins of less than 16 kDa makes determining relative amounts of these polypeptides impossible.

Terminal *BglI* restriction fragments localize T5 pre-early genes. To localize pre-early functions, the restriction enzyme *BglI* was used to excise the 6.3-kb fragment from the left end of the genome and the 3.9-kb fragment from the right end (Fig. 1). *BglI* has restriction sites closer to each terminus than *SalI*, making it an apt choice to localize genes in a smaller region within the *SalI* pieces. The results of cell-free transcription and translation of both the 6.3- and 3.9-kb fragments are shown in Fig. 4. The electrophoretic patterns show clearly that most of the bands present in the *SalI* fragments from either wild-type T5 or an A2 mutant are also present in the left 6.3-kb *BglI* fragment, including the proteins identified as A1, A2, and *dmp*. Although resolution of polypeptides is poor below 16 kDa, it is apparent that most of these smaller polypeptides are also encoded by this *BglI* fragment. However, the prominent band at 40 kDa is missing and is replaced by a series of minor bands of slightly smaller molecular sizes. This fact may indicate that *BglI* either cuts within the 40 kDa protein or separates it from a promoter, resulting in transcriptional starts from promoterlike regions proximal to this protein not used when the normal promoter is functional. Another minor 23-kDa protein is also present in the complete left and right pre-early regions, yet it is missing from the 6.3- and 3.9-kb subsections of the pre-early region.

Although the 6.3-kb left-region *BglI* fragment codes for virtually all major and minor bands encoded by the 13.2-kb *SalI* fragment, the 3.9-kb *BglI* fragment from the right end induces only two very minor bands corresponding to low-molecular-weight polypeptides, though similar amounts of DNA were used in comparing the two *BglI* fragments.

FIG. 5. Map locating T5 pre-early and neighboring genes with restriction fragments examined in this study. The left and right portions of the viral genome are positioned to emphasize the directly repeated nature of the terminally redundant (TR) regions and to demonstrate which regions of the 13.2- and 15.2-kb *SalI* fragments are not common to both. The numbers above and below the left and right ends of the genome are the sizes of restriction fragments in kilobases, while R, B, and S indicate, respectively, the locations of *EcoRI* (see Discussion and reference 4), *BglI*, and *SalI* sites in this region of the phage genome. p* and p** designate approximate locations of two major pre-early promoters.

Because of the faintness of these bands, it is not clear whether these two polypeptides represent bona fide pre-early proteins. Neither the 3.9- nor the 6.3-kb *BglI* fragment, containing exclusively pre-early DNA, encodes the presumed 30-kDa early protein.

These results show that the pre-early proteins are positioned compactly within a small part of the T5 genome bordered by regions several kilobases in size which evidently do not encode proteins. These results also confirm, at the protein level, the similarity of the direct terminal repeats. Figure 5 summarizes, schematically, what these data demonstrate about the arrangement of the pre-early sections, with the two termini positioned for ready comparison. The probable positions of the 40-kDa protein disrupted by *BglI* digestion and of the 30-kDa protein in the early region are shown, as are additional relevant features of the pre-early region such as likely promoter locations (20) which may control the polypeptides positioned by the evidence presented here.

DISCUSSION

By using isolated restriction fragments from the genome of bacteriophage T5 as templates for cell-free transcription and translation, it has been possible to localize essentially all pre-early proteins, including the A1, A2, and *dmp* gene products, to a 6.3-kb section at either end of the genome. The direct repeats at each end also contain at least 3.9 kb of DNA which appear not to encode any major polypeptides under these conditions. These empty regions lie immediately to the right of the protein-coding portions of the genome. Thus, it has been possible to link, generally, physical and classical genetics maps for the T5 pre-early region. The paucity of genes in this region of the terminal redundancy is consistent with the observation that this region is unessential and deletable in the closely related phage, BF23 (19).

The convenient location of *SalI* restriction sites at just three points along the T5 genome has allowed separation of

both terminally redundant regions. Past research indicated that the redundancies were identical (17), though neither end had been sequenced. The gel patterns produced by the 13.2- and 15.2-kb *SalI* fragments in the cell-free system confirm that the same polypeptides are encoded by both ends. Besides pre-early genes, both restriction fragments include flanking portions of the genome, of about 3 and 5 kb for the left and right *SalI* fragments, respectively. Knowing that all polypeptides except one are common to both fragments, it is possible to deduce the probable location of the exceptional protein from the left fragment as being within the 3 kb to the right of the left pre-early region. Comparison of the cell-free protein patterns to wild-type infection patterns shows a similar protein appearing during the pre-early to early transition, indicating that the exceptional protein may indeed be an early peptide from the C2 region of the T5 genetic map. This region is believed to encode the C2 gene product, an activity instrumental in regulating synthesis of early and late viral RNA species. However, the apparent molecular weight of this gene product is about 95,000 (6), considerably more than that of the polypeptide observed in this work.

Under the same conditions, the 5-kb region to the left of the right pre-early region induced no detectable polypeptides, indicating either that this region is empty or that any coding regions have been separated from their promoters. It is also possible that pre-early genes within the 15.2-kb *SalI* fragment are expressed in preference to any late genes present, because late-gene transcription requires phage-modified host RNA polymerase. The product of the D15 gene also seems important for late-gene transcription (11). If this region does encode few proteins, though, it is further evidence that pre-early, early, and late regions are separated from one another by noncoding regions, at least partly explaining the maximal recombination observed between any given marker in one region and all markers from another region.

Using *BglI* to subdivide the pre-early region, we found

that a 6.3-kb region encoded most pre-early proteins. Transcriptional control regions must lie in this section too. Previous studies also have positioned the A1 and A2 genes within an area included by the 6.3-kb fragment (4, 5). Using a T5 mutant with additional *EcoRI* sites in the pre-early region, Brunel and Davison (4) found that the A2 and A1 genes were located in a 1.7-kb section between 2,500 and 4,200 base pairs from the left end of the phage. In contrast to the 6.3-kb fragment, the 3.9-kb fragment encodes nothing significant by the cell-free system. As pointed out above, a major band at 40 kDa is present in both the 13.2- and 15.2-kb *SalI* fragments, yet the band is absent in both *BglI* fragments, indicating that this restriction site either cuts the protein or separates it from essential control sequences. Clearly, the 3.9-kb fragment does not contain promoters necessary for transcription of the A1, *dmp*, or A2 gene, because these genes continue to be made by isolated *BglI* 6.3-kb fragments.

The relative intensities of the A1, *dmp*, and 40-kDa bands expressed in the cell-free system within a given lane are similar, indicating that these three genes are controlled either by a single promoter or by promoters of approximately the same strength. Earlier research led to the assignment of three promoters to each pre-early region (20), where the two strongest appear to be situated near the *BglI* site (Fig. 5). Based on their proposed locations and the results of *BglI* digestion on cell-free gene expression patterns, it seems probable that the leftmost of these two promoters controls transcription of all genes found in the 6.3-kb *BglI* fragment, and therefore the direction of transcription is from right to left in this region. This possibility is consistent with past work indicating that the direction of transcription is towards the left end of the phage, through, in order, the three pre-early genes A3, A2, and A1 (7, 14). Its neighbor to the right could control transcription of the gene for the 40 kDa protein, but either the promoter or the gene itself is disrupted by restriction.

An apparent scarcity of genes at the far right end of the left pre-early region might be expected because of the two-step entry of the T5 genome into its host. As the genes included in the 6.3-kb fragment enter the cell, part of the 3.9-kb fragment may remain complexed or at least masked by *E. coli* membrane components and therefore be inaccessible.

Assuming an average molecular weight of 110 for an amino acid, the three most prominent discrete bands encoded by the 6.3-kb fragment at 65, 27, and 16 kDa, i.e., the putative A1, *dmp*, and A2 proteins, respectively, require half its coding capacity. This capacity does not include promoters or peptides with sizes smaller than 14 kDa, which are unresolvable yet clearly present on these 10% sodium dodecyl sulfate gels. As with many other viruses, T5 has compressed large amounts of information into small parts of its genome.

The large number of faint minor bands observed in the cell-free *BglI* and *SalI* patterns may be caused by transcriptional starts at promoterlike sequences or by inappropriate termination, because the combined total of their weights vastly exceeds the coding capacity of these fragments. These minor bands are seen in both cell-free and in vivo gel patterns, demonstrating they are not simply artifacts of the in vitro system. Possibly the single-chain nicks (1) found at five principal sites (80 to 90% of a given population) and many minor sites (less than 20% of a population) in the T5 genome are in some way responsible for these hypothetical transcriptional misstarts or stops. In this context, several minor nicks have been shown to cluster in the T5 pre-early region (18).

Judicious isolation of restriction fragments in the pre-early regions of bacteriophage T5 has allowed dissection of the genome and assignment of several known pre-early functions to a small section of the T5 physical map. Furthermore, the information presented here is consistent with predicted pre-early promoter locations (20) and demonstrates that the sequences controlling expression at almost all pre-early genes are within the same 6.3-kb fragment as the genes themselves. Thus, this method has proved convenient for focusing on the pre-early region at T5, a portion of the phage not readily probed by other techniques.

ACKNOWLEDGMENTS

This work was supported by research grant PCM 8215784 from the National Science Foundation. P. Blaisdell was supported by National Institute of Health grant T32-GM07323.

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