Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 4937–4941, August 1982
Biochemistry

Nucleotide sequences involved in bacteriophage T4 gene *32* translational self-regulation

(gene expression/translation initiation/helix-destabilizing protein/autoregulation/protein fusion)

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Communicated by Peter H. von Hippel, May 17, 1982

ABSTRACT **We have determined the nucleotide sequence of a cloned segment of the bacteriophage T4D chromosome, which contains the regulatory sequences and the structural gene (gene *32*) for the single-stranded DNA binding protein (gp32). The amino acid sequence predicted by translation of the structural gene agrees well with that published for gp32 [Williams, K. R., LoPresti, M. B., Setoguchi, M. & Konigsberg, W. H. (1980) *Proc. Natl. Acad. Sci. USA* 77, 4614–4617]. To localize the nucleotide sequence involved in translational self-regulation of gene *32*, we have constructed a series of plasmids in which gene *32* is fused to an amino-terminal deletion mutant of the β-galactosidase gene of *Escherichia coli*. Expression of a β-galactosidase fusion protein that contains only the first seven amino acids of gp32 is still repressed by gp32. The ribosomal binding site of gene *32* is flanked by a repetitive A+T-rich sequence. Preferential and cooperative binding of gp32 to this region of its mRNA could inhibit translation initiation and, thus, would account for the autoregulation.**

The helix-destabilizing gene *32* protein of bacteriophage T4 (gp32) has a multifunctional role (1): replication (2), recombination (3), and repair (4) of the viral chromosome all require its activity. The expression of gene *32* is self-regulated, and control operates at the level of translation rather than transcription (5–10). As a first step to a better understanding of the mechanism by which gp32 inhibits translation of its own mRNA, we isolated a hybrid plasmid that contains gene *32*. Because functional gp32 has deleterious effects on the host cell, the plasmid we constructed contains a mutant (*32amA453*) DNA sequence (11, 12). Using DNA prepared from this plasmid and its deletion derivatives, we have determined the complete nucleotide sequence of a segment of the T4 DNA that includes gene *32*. Studies with a series of deletion derivatives of the plasmid suggest that the nucleotide sequence in the vicinity of the gene *32* initiation codon is involved in translational repression.

MATERIALS AND METHODS

Bacteria and Plasmid Strains. Strain HB101 (F^- *pro leu lacY* str^r *hsdS endA recA*$^-$ *su*II$^+$) used for bacterial transformations by plasmid DNA was obtained from W. Arber. The chromosomal *lac* deletion strain MC1061 (*araD139*, Δ(*ara–leu*)*7697*, Δ*lacX74*, *galU*$^-$, *galK*$^-$, *hsr*$^-$, *hsm*$^+$, *strA*) used for studies of gene *32*–β-galactosidase gene fusion plasmids was provided by M. Casadaban.

The gene *32* plasmids used were made from pKSK12A, a pBR322 derivative containing a 2,600-base-pair (bp) *Hin*dIII segment of T4 DNA that includes genes *59* and *32* and a portion of gene *33* (11, 12). gp32 fusions with β-galactosidase were constructed by using pMC1403, a pBR322 derivative that contains most of the *lac* operon but is deleted for the *lac* promoter and the first six codons of the *lacZ* gene. Inserted immediately prior to the *lacZ* gene is a synthetic linker sequence that contains the recognition sites, unique in the plasmid, for the restriction enzymes *Eco*RI, *Sma* I, and *Bam*HI (13).

DNA Restriction Enzyme Analysis; Construction of Deletion Derivatives of Gene *32* Plasmids. Recombinant DNA techniques were those previously used in this laboratory (11, 12, 14). BAL 31 nuclease digestions were performed under the conditions described by Gray *et al.* (15).

Nucleotide Sequence Analysis. The methods used were those of Maxam and Gilbert (16). The strategy of restriction fragment end labeling is given in the legend to Fig. 2*B*.

Coupled *in Vitro* Transcription–Translation System with Plasmid DNA as a Template. *Escherichia coli* strain D10 su$^-$ (17) was used to prepare cell-free extracts as described by Zubay *et al.* (18) with minor modifications (19). Except as noted in the figure legends, the ionic conditions of the reaction mixture, including contributions from the extract, were identical to Greenblatt *et al.* (ref. 19; see ref. 20 for additional details). In this system, repression by gp32 requires a somewhat higher gp32 concentration than that reported by Lemaire *et al.* (10). This could reflect differences in the S30, the ionic conditions, the activity of the gp32 preparations, or gp32-binding species (either RNA or DNA) in the extracts (see ref. 10).

RESULTS AND DISCUSSION

Complete Nucleotide Sequence of a 1,340-bp *Msp* I–*Hin*dIII Fragment Containing Gene *32*. The nucleotide sequence of T4 gene *32* was established from deletion derivatives of pKSK12A, a hybrid plasmid carrying the gene cloned in pBR322 (11, 12). The initial sequence determination utilized a complete *Alu* I restriction map of the gene *32* region of the T4 chromosome that we had constructed (11). This map, when aligned with the genetic map, indicated that the initiation codon of gene *32* was located in a 450-bp *Alu* I fragment; this was confirmed by nucleotide sequence analysis. The coding sequence (21) starts 65 bp in the 3′ direction from the *Alu* I site (Fig. 1). The complete nucleotide sequence of a region of the T4 chromosome that includes gene *32* is shown in Fig. 2*A*. The strategy used to obtain this sequence is shown in Fig. 2*B*.

The nucleotide sequence in the vicinity of the gene *32* initiation codon has several striking features which, in view of the translational self-regulation of this gene, may be of particular interest (Figs. 1 and 2*A*). A Shine–Dalgarno sequence, which has a five-bp complementarity to the 3′ end of the 16S ribosomal RNA (22), precedes the initiation codon by seven bases. This presumed ribosomal binding site is in the middle of a 40-bp sequence that is exceptionally A+T rich (92%); apart from the G in the initiation codon and the two Gs in the ribosomal

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Abbreviations: gp32, gene *32* protein; gp*rIIB*, gene *rIIB* protein; bp, base pairs; kDal, kilodalton.

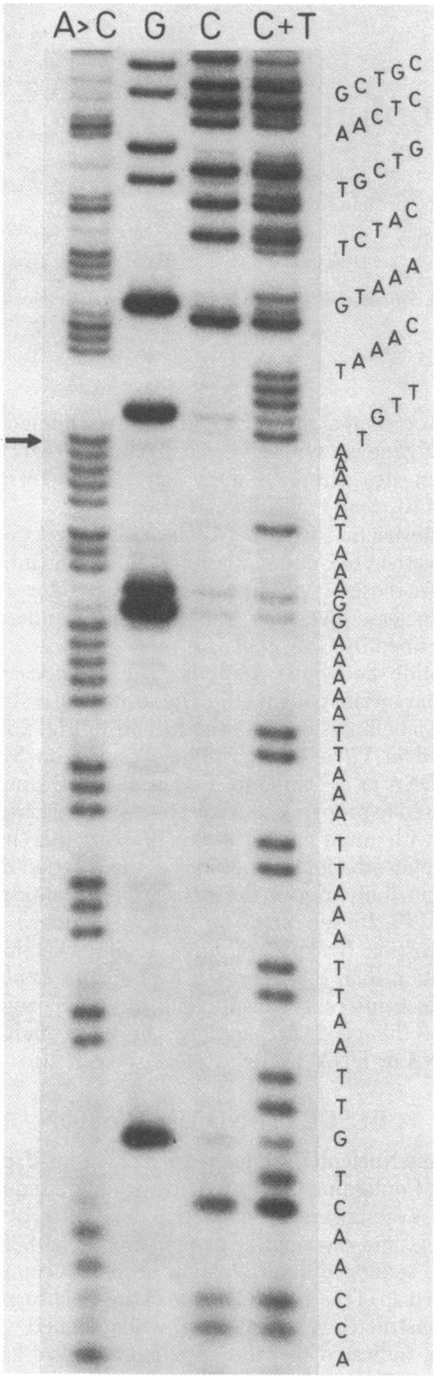

FIG. 1. Autoradiograph of 8% polyacrylamide DNA sequence analysis gel: the nucleotide sequence in the region of the initiation codon of gene *32*. A 437-bp *Alu* I fragment obtained from plasmid pMJK4-2 (12) was end-labeled with polynucleotide kinase and then cut with *Hin*fI, and the 314-bp fragment was purified. The segment of the sequence shown, which includes the A+T region in the vicinity of the gene *32* initiation codon (indicated by an arrow), starts at base pair −39 and ends at base pair +35 in Fig. 2.

binding site, the sequence consists exclusively of alternating runs of As and Ts. The pentanucleotide (T-T-A-A-A) occurs four times—three times in tandem immediately prior to the ribosomal binding site and once immediately after the initiation codon. Cooperative binding of gp32 to this sequence on the gp32 mRNA could impede the formation of a translation initiation complex. Because gp32 covers five to seven bases in binding to nucleic acids (either DNA or RNA) (23), it is possible that the binding to the five-base repeated sequence is specific. Recently, von Hippel *et al.* (24) have suggested that even modest differences in intrinsic binding specificity of gp32 could be amplified by cooperativity of protein binding. Another relevant feature of this portion of the mRNA may be a predicted lack of stable secondary structure (unpublished observations; and ref. 24), making it a preferred location for gp32 binding.

The published amino acid sequence of gp32 (21) is in good agreement with that predicted from the nucleic acid sequence. The two sequences differ by 11 out of 301 amino acids. One of the differences reflects the fact that we cloned a nonsense mutant rather than the wild-type gene *32* (see the legend to Fig. 2). With one exception (Ser at position 86), the remaining inconsistencies involve interchange of Asp and Asn (five times) or Gln and Glu (four times) residues. In amino acid analysis, discrimination of these pairs of amino acids is difficult and is a common source of error.

Hybrid plasmids that contain the T4 segment from the *Msp* I site (−343 in Fig. 2) to beyond gene *32*, efficiently express the gene from a promoter located within the insert. A study of a series of deletion derivatives of these plasmids shows that viral sequences essential for gene *32* transcription initiation in uninfected cells are located less than 200 bp prior to the initiation codon (unpublished observation). In T4-infected cells, the major gene *32* transcript is ≈200 bp larger than that necessary to encode the gene (9). Thus, a gene 32 promoter recognized after infection must also lie within the sequence shown in Fig. 2*A*. However, the synthesis of this gene *32* monocistronic mRNA depends upon the presence of the T4 *mot* gene product (25). Therefore, this transcript would perhaps not be expected in uninfected plasmid-containing cells that lack the *mot* product. It will be important to map precisely the start points of gene *32* plasmid transcripts made in uninfected cells and compare them with those obtained after phage infection.

Beyond gene *32*, 40 bp after the tandem termination codons, is a sequence that closely resembles the consensus (26) transcription-termination sequence (a stem loop structure followed by a run of Ts).

The Nucleotide Sequence Involved in the Repression of Gene *32* mRNA Translation. The interaction between gp32 and its mRNA results in translational repression. To identify the sequences involved in this repression, we examined deletion derivatives of the gene *32* plasmid. These studies are illustrated by the comparison of the two plasmids pMJK4-20 and pKH13. Plasmid pKH13 contains only the first 126 of the 301 amino acids in gene *32* (Fig. 3). *In vitro* experiments, in which DNA of the two plasmids were used as template in a coupled transcription–translation system, indicate that the synthesis of the gene *32* nonsense fragment (115 amino acids) is identical for both plasmids. When purified gp32 was added to the two reactions, the synthesis of the gene *32* nonsense fragment could be repressed almost completely in each case (Fig. 4). Thus, the deletion in pKH13 does not affect the control of gene *32* expression. That translational repression is obtained in this system was shown directly in experiments with the *in vitro* system in which transcriptional and translational effects were analyzed separately (data not shown).

Fig. 4 also confirms the specificity of gp32 translational repression; a concentration of gp32 sufficient to effectively repress expression of gene *32* from the plasmid has little effect on the pattern of protein synthesis directed by pBR322 or pTB10, a plasmid which contains the amino-terminal portion of gene *rIIB* protein (gp*rIIB*) (14). The translation of this T4 gene, whose pattern of transcription in infection is similar to that of gp32, is insensitive both *in vivo* (5) and *in vitro* (10) to the level of gp32. The sequence flanking the ribosomal binding site of

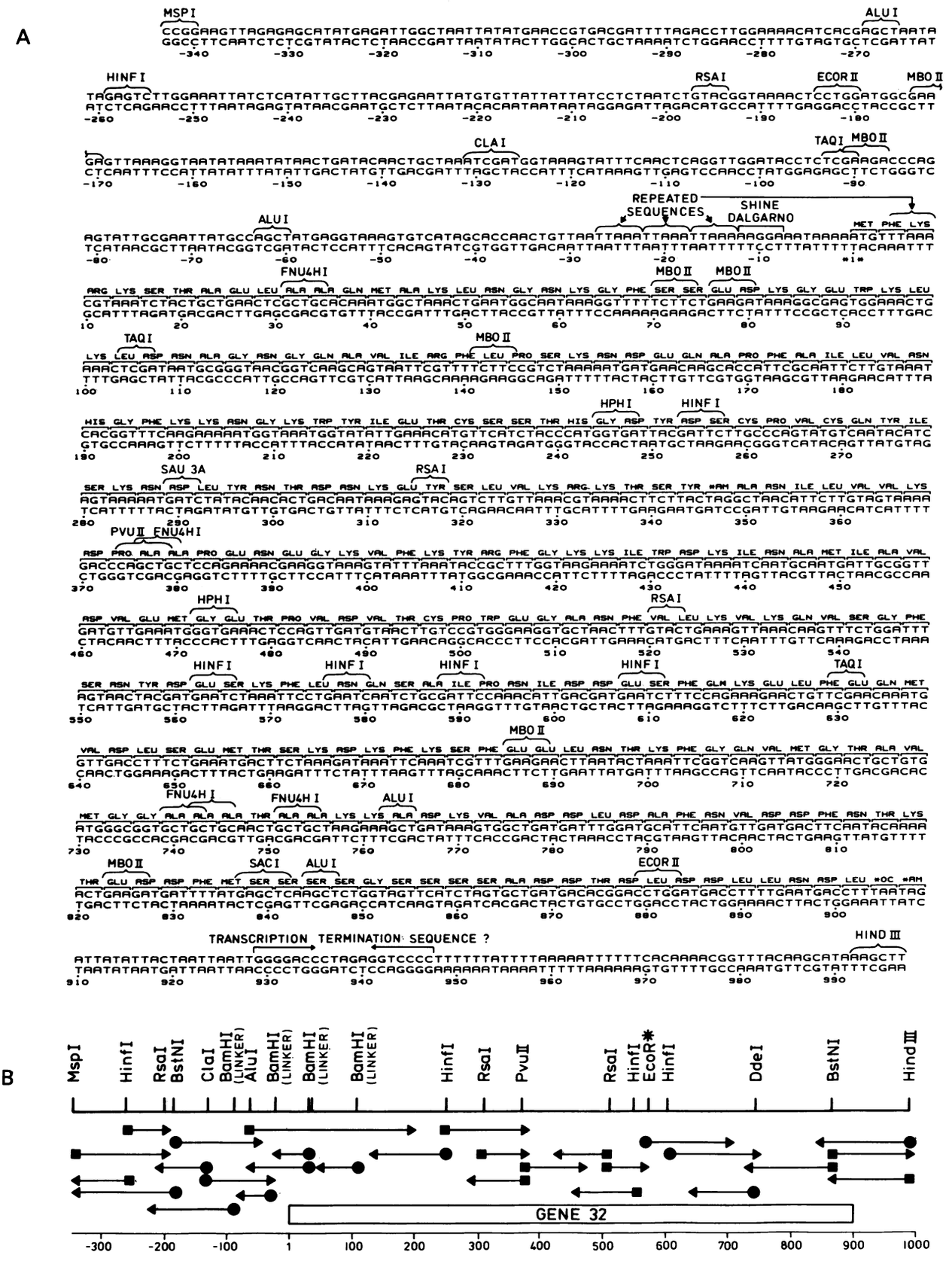

FIG. 2. (*A*) Nucleotide sequence of a 1,340-bp *Msp* I–*Hin*dIII fragment of the T4 chromosome that includes gene *32*. The coordinates of the nucleotide sequence have been aligned so that the gene *32* initiation codon starts at nucleotide number 1. The sequence is that of the mutant *32amA453*, which results in the amber codon (UAG) at the position of amino acid 116. In the wild-type sequence (21), amino acid 116 is a tryptophan (UGG). An open reading frame of 96 amino acids that terminates 59 bp prior to gene *32* corresponds to the carboxy terminus of the 26-kDal protein, which is probably the gene *59* product. (*B*) Nucleotide sequence analysis strategy. The strands were labeled at the 5′ (■) or 3′ (●) ends by polynucleotide kinase or by filling in with the Klenow fragment of polymerase I and then submitted to base-specific cleavage reactions. The extent to which the sequence could be read with confidence is indicated by the length of the arrows.

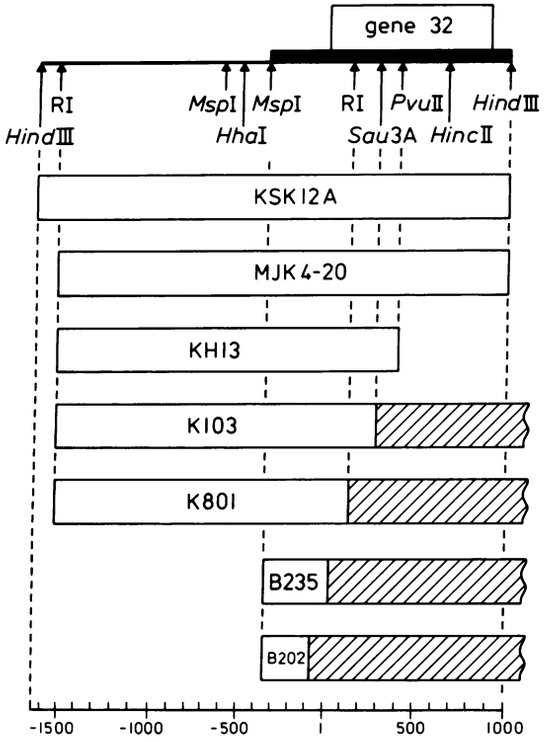

FIG. 3. Physical map of the phage DNA insert in plasmid pKSK12A and of its deletion derivatives. The portion of the pKSK12A insert that has been sequenced is indicated by a thick black line, and the location of gene *32* is also marked. The segment of the pKSK12A insert contained in the various deletions is indicated by the extent of the open box containing the plasmid name. Those plasmids which have been fused to the *lacZ* gene of pMC1403 are indicated by a hatched box beginning at the site of fusion. The construction and properties of pKSK12A and pMJK4-20 have been described (12). Plasmid pKH13 was obtained by digestion of pMJK4-20 with *Pvu* II followed by blunt-end ligation to recircularize. A purified 1,950-bp *Eco*RI–*Sau* 3A fragment was obtained from pKH13 and inserted into *Eco*RI- and *Bam*HI-digested pMC1403. The resulting plasmid (placK103) has an in-phase fusion between the codons corresponding to amino acid 97 of gp32 and amino acid 7 of β-galactosidase. The plasmid placK801 was obtained by partial digestion with *Eco*RI* (27) of the *Eco*RI–*Sau* 3A fragment, followed by ligation into pMC1403 linearized with *Eco*RI. The resulting in-phase gene *32*–β-galactosidase gene fusion at base pairs corresponding to amino acid 45 of gp32 (confirmed by nucleotide sequence analysis) confers a strong lac^+ phenotype. The plasmids placB235 and placB202 were obtained with the exonuclease BAL 31. In essence, we constructed a pMC1403 derivative in which the T4 insert at the *Sma* I site extended from the *Msp* I site (−343) to the *Fnu*4H1 site (+31) in Fig. 2. This plasmid was linearized at its unique *Bam*HI site and digested to various extents with BAL 31. The DNA was then digested with *Pst* I and the *Pst* I–BAL 31 fragment ligated into pMC1403 cut with *Pst* I and *Sma* I. Transformants of strain MC1061 giving blue colonies on 5-chloro-4-bromo-3-indolyl-β-D-galactoside plates were examined for appropriately sized inserts. The end points of both of these plasmids were determined by nucleotide sequence analysis from the *Bam*HI site. Between the gene *32* initiation codon and the *Bam*HI site of placB235, the sequence was determined to be A-T-G-T-T-T-A-A-A-C-G-T-A-A-A-T-C-T-A-C-T-G-G-G-G-A-T-C-C. The sequence of *lacZ* beyond the *Bam*HI site is G-G-A-T-C-C-C-G-T-C-G-T-T-T-T-A (13).

gprIIB is known (28) and has no repeated A+T-rich sequence similar to gene *32*.

In the reaction using the plasmid pMJK4-20 as a template, even at low concentrations of gp32, several minor protein species as well as the gene *32* amber fragment are strongly repressed by gp32 (Fig. 4 *A* and *E*). With one exception, these proteins can be shown to be read-through peptides of the gene *32* nonsense mutation (the S30 was prepared from a su^- strain): they react with antibody to purified gp32 (unpublished observation) and are abolished by deletion of gene *32* sequences 3′

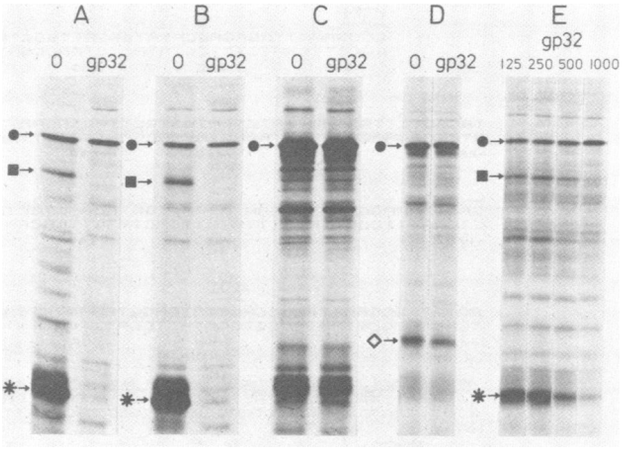

FIG. 4. Autoradiogram of a 15% sodium dodecyl sulfate/polyacrylamide gel of proteins synthesized *in vitro* with hybrid plasmids as template: specific repression of gene *32* expression by gp32. Plasmids pMJK4-20 (lanes A and E), pKH13 (lanes B), pBR322 (lanes C), and pTB10 (lanes D) were used as templates for a Zubay system S30. The reactions were carried out at 37°C for 60 min. In the 25-μl reaction mixtures, plasmid DNA was present at 20 μg/ml, Mg^{2+} at 14 mM, and cAMP at 2 mM. Other components of the reaction were as described in ref. 19. The reactions marked "O" contained no gp32 whereas those marked "gp32" contained gp32 (purified fraction 32.7F provided by R. L. Burke and B. Alberts) at 800 μg/ml. The different amounts of gp32 added to the E reactions are indicated in μg/ml. Reactions D and E did not contain cAMP. *, The position of *32amA453* (11 kDal); ●, the vector plasmid β-lactamase (31 kDal); ■, the 26-kDal protein, which may be the product of T4 gene *59*; and ◇, the gprIIB peptide.

to *32amA453* (Fig. 4). The significant exception is a 26-kDal protein that, by deletion mapping (see the legend to Fig. 5), is 5′ to gene *32* in a region identified genetically (11, 12) as containing phage T4 gene *59*. It is known (9) that, in T4-infected cells, one gene *32* transcript is sufficiently large that it could start prior to the 26-kDal protein (gene *59* product ?) and still encode gene *32*. One possible explanation of this joint repression is that preferential and highly cooperative binding of gp32 to the gene *32* translational regulatory sequence could result in inhibition of translation of the upstream gene as well as the downstream one.

Additional deletion derivatives of the gene *32* plasmid should further delimit the nucleotide sequence involved in the control function. The shorter the coding region, however, the more difficult it becomes to quantitate the synthesis of gene *32*-related peptides. To overcome this problem, we have fused gene *32* to the Z gene of the lactose operon present in a plasmid constructed by M. Casadaban (13). In this plasmid (pMC1403), the operon is not expressed because the *lac* promoter, the operator, and the first six amino acids of the *lacZ* gene are deleted. The beginning of the *lacZ* gene in this plasmid is immediately preceded by a synthetic DNA linker molecule with sites for *Eco*RI, *Sma* I, and *Bam*HI. Insertion into any of these restriction sites of a segment of DNA with a promoter and a translation-initiation sequence (in the proper reading frame) results in a lac^+ phenotype in a host with a chromosomal *lac* operon deletion.

Using this plasmid, we have constructed a series of gene *32*–β-galactosidase gene fusion plasmids with progressively smaller segments of the gene *32* coding sequence. The left-hand end of the gene *32* insert includes, in all cases, a good promoter for gene *32* transcription. The restriction endonucleases *Sau* 3A and *Eco*RI* allowed us to obtain gene *32*–β-galactosidase gene fusions that encode, respectively, 97 and 45 amino acids of gp32 (placK103 and placK801, Fig. 3). The *in vitro* expression of the gp32–β-galactosidase fusion protein from both of these plasmids is specifically repressed by gp32 (Fig. 5). To confirm the

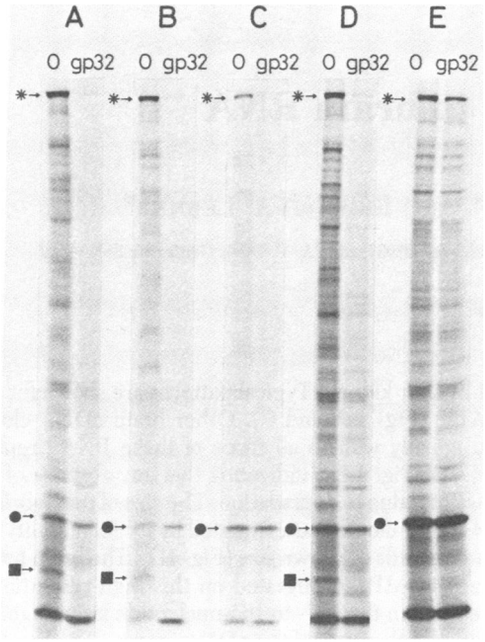

FIG. 5. Autoradiogram of a 10% sodium dodecyl sulfate/polyacrylamide gel of protein synthesized *in vitro* by using various gene *32*–β-galactosidase gene fusion plasmids as templates: specific repression of fusion protein expression by gp32. The [^{35}S]methionine-labeled proteins were synthesized by an S30 system primed by various gene *32*–β-galactosidase gene fusion plasmids: lane A, placK103; lane B, placK801; lane C, placB235; lane D, placK103; and lane E, placB202. The structure of the plasmids is described in Fig. 3. The 25-μl reactions were as described in the legend to Fig. 4 except that plasmid DNA was present at 10 μg/ml and gp32 (where present) at 1 mg/ml (lanes A, B, and C) or 0.8 mg/ml (lanes D and E). After incubation at 37°C for 30 min, the reactions were stopped by adding sample buffer, and the mixtures then were boiled for 2 min. *, Position of the fusion proteins; ●, position of β-lactamase; and ■, position of the 26-kDal protein. The deletion 5′ to the gene *32* sequence in plasmid placB235 and placB202 results in the loss of the ability to direct the synthesis of the 26-kDal protein.

generality of this result, we also fused the gene *32* insert in placK103 to the structural gene for tetracycline resistance of pBR322; expression of the gp32–tetracycline resistance fusion protein is specifically repressed by gp32 (data not shown).

The minimal sequences involved in translational control can be further circumscribed with the exonuclease BAL 31. Starting with a gene *32*–β-galactosidase gene fusion at the *Fnu*4HI site corresponding to amino acid 11 of gp32, we have generated a number of deletions in the direction of the initiation codon. The sequences of several of these have been determined to define precisely the end point of the deletion. The plasmid placB235 extends from the *Msp* I site at the beginning of the sequence shown in Fig. 2 to base pair 21 of gene *32* and results in an in-phase fusion with the β-galactosidase gene. *In vitro* experiments (Fig. 5) demonstrate effective repression of synthesis of the gp32–β-galactosidase fusion protein by purified gp32. In contrast, the plasmid placB202 is relatively insensitive to repression by gp32. This plasmid has the same left end as in placB235, but the BAL 31 digestion was more extensive; the plasmid ends 86 bp prior to gene *32*, resulting in an in-phase fusion of the β-galactosidase gene to the long open reading frame ahead of gene *32*, which may correspond to gene *59*. It is unclear where the translation that produces this fusion protein initiates. From the size of the protein, it must be rather close to the point of fusion, perhaps at the in-phase ATG codon at position −128.

A comparison of the plasmids placB235 and placB202 shows that a sequence essential to specific repression by gp32 must lie in the segment between the different end points of these two plasmids. The ribosomal binding site of gene *32*, flanked by the repeated A+T-rich sequence, lies within this segment. As we have noted above, preferential and cooperative binding of gp32 to this region on the mRNA could block translation initiation by sequestering the ribosomal binding site. Thus, competition for available gp32 between this sequence on the gene *32* mRNA and single-stranded DNA or other binding sites would determine the level of gene *32* translation (5, 6).

We are grateful to all of our colleagues at the University of Geneva and Biogen who contributed to this work with aid, advice, and discussion. The technical assistance of J. Poitry in the nucleotide sequence determinations was invaluable. This work was funded in part (H.M.K.) by a grant from the Swiss National Science Foundation (No. 3.078.81) to R. H. Epstein, whose aid, interest, and support was essential. Additional support and facilities (B.A.) were provided by Biogen S. A. (Switzerland).

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