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IN VITRO SYNTHESIS OF A PEPTIDE WHICH MODIFIES THE TRANSCRIPTIONAL SPECIFICITY OF BACILLUS SUBTILIS RNA POLYMERASE

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Summary

After infection of Bacillus subtilis by phage SP82 the sigma subunit of RNA polymerase is replaced by a positive regulatory subunit of 28,000 molecular weight (the 28K peptide) thereby altering the specificity of transcription. The synthesis of messenger RNA coding for the 28K peptide has been investigated using in vitro translation followed by chromatography of the translation products on columns of RNA polymerase immobilized on Sepharose. In vivo, synthesis of the mRNA occurred by 4 min after infection and was not affected by addition of chloramphenicol just prior to infection. In vitro, synthesis of the 28K peptide was detected in experiments using a coupled transcription-translation system derived from B. subtilis.

Transcription of the genome of Bacillus subtilis phage SP82 (1,2) and the related phage SP01 (3) is regulated largely by sequential modification of the host RNA polymerase. The first modified form, isolated from cells 8 min after infection, has the same composition as the host enzyme except that the sigma subunit is missing and there are two additional peptides of 28,000 (28K) and 18,000 (18K) molecular weight (2,4). A number of studies (1,3,5,6) have shown that the presence of the 28K peptide is required for the transcription of "middle genes"; the functions of the 18K peptide have not been determined.

The mechanisms involved in the replacement of the sigma subunit of the host polymerase by the 28K and 18K peptides are not known. If the mRNA directing the synthesis of the 28K peptide is an "early RNA", it would be expected that this transcript would be produced by in vitro transcription of SP82 DNA by the host polymerase and that its synthesis could be detected by translation. In the present study, we have investigated the in vivo and in vitro synthesis

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of mRNA coding for the 28K peptide by chromatography of *in vitro* translation products on columns of RNA polymerase immobilized on Sepharose.

Materials and Methods

Isolation of SP82, DNA, RNA and RNA polymerase. Previously described procedures were used for phage infection, the preparation of SP82 DNA, the isolation of RNA from SP82-infected *B. subtilis* and the preparation of RNA polymerase (2,7). Synthesis of RNA *in vitro* was performed according to Hiatt and Whiteley (4) in the presence of 0.15 M NaCl.

Cell-free protein synthesis, gel electrophoresis and autoradiography. Uncoupled protein synthesis was measured as described (4) using S-30 fractions from *E. coli* MRE 600 (8) or *B. subtilis* SR22 (9). Coupled RNA and protein synthesis (4) utilized rifampin-resistant RNA polymerase isolated from *B. subtilis* strain OSB 420 (10). Sodium dodecyl sulfate (SDS) gel electrophoresis and autoradiography were performed as described earlier (4).

RNA polymerase-Sepharose columns. The procedures of Ratner (11) was used to link RNA ploymerase isolated from uninfected *B. subtilis* to Sepharose 4B activated according to March et al. (12). Samples containing labeled proteins were adsorbed to 0.1-0.2 ml columns for 1 to 2 hours and washed with 1 M Nacl. The beads were extruded into the SDS-containing sample buffer used for gel electrophoresis, heated in a boiling water bath, centrifuged to remove the beads and the supernatants were applied to SDS-polyacrylamide slab gels for electrophoresis and autoradiography.

Results

Synthesis of the 28K peptide by translation of RNA produced in vivo. Our studies have shown that replacement of the sigma subunit by the 28K peptide is virtually complete by 8 min after infection (2) and that the 28K peptide is synthesized from the 3-6 min to the 9-12 min intervals after infection (Milhausen, unpublished). It is reasonable, therefore, to propose that mRNA directing the synthesis of this peptide is an early RNA, synthesized by the unmodified host polymerase. Previous investigations showed that when RNA produced *in vitro* by this enzyme from SP82 DNA was incubated with an *E. coli* translation system, approximately 15 peptides were synthesized (4). However, none of the translation products had the electrophoretic mobility of the 28K peptide.

Three possible explanations for this observation may be proposed: 1) the unmodified *B. subtilis* polymerase does not produce mRNA coding for the 28K peptide *in vivo* -- i.e., modification of the enzyme is required, 2) the *E. coli* cell-free extract is not capable of translating the mRNA coding for the 28K

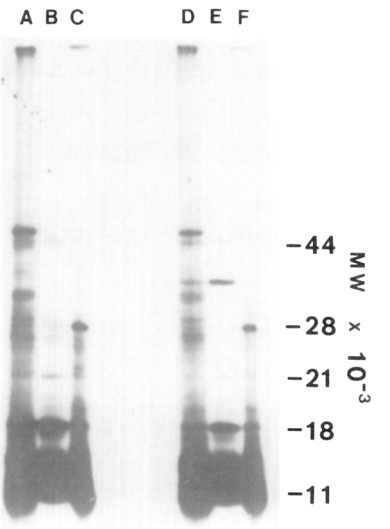

Figure 1. Autoradiogram resulting from the one-dimensional SDS-polyacrylamide electrophoresis of peptides synthesized in vitro from RNAs produced in vivo and subsequently adsorbed to columns of RNA polymerase-Sepharose.

Lane A, total peptides produced by translating RNA extracted from SP82-infected, chloramphenicol treated cells ("CM-RNA") as described in the text; lane B, peptides produced by translating CM-RNA and eluted from a column of RNA polymerase-Sepharose by 1 M NaCl; lane C, peptides produced by translating CM-RNA and eluted from a column of RNA polymerase-Sepharose by SDS; lane D, total peptides produced by translation of RNA extracted from B. subtilis 4 min after infection ("4 min RNA"); lane E, peptides produced by translation of 4 min RNA and eluted from a column of polymerase-Sepharose by 1 M NaCl; lane F, peptides produced ty translation of 4 min RNA and eluted from a column of polymerase-Sepharose by SDS.

peptide or 3) the 28K peptide is produced in limiting amounts and thus cannot be detected by SDS gel electrophoresis.

The first two possibilities were tested by translating two RNA preparations: the first was extracted from cells 4 min after infection and the second was isolated from chloramphenicol-treated cells 4 min after infection (the chloramphenicol was added 1 min prior to infection). The translation products were chromatographed on columns of RNA polymerase immobilized on Sepharose and eluted with 1 M NaCl to remove loosely bound peptides and then with SDS to remove tightly bound peptides. The two eluted fractions were electrophoresed on a one-dimensional SDS slab gel and detected by radioautography. As shown in lanes C and F of Fig. 1, a band having the mobility of the 28K peptide was pre-

Table 1: Incorporation of ^{35}S-methionine by extracts of *E. coli* and *B. subtilis*.

Translation	Extract from	Added Component	cpm ^{35}S-methionine*
Uncoupled	*E. coli*	- RNA*	31,000
	E. coli	+ RNA*	326,000
	B. subtilis	- RNA*	20,000
	B. subtilis	+ RNA*	207,000
Coupled	*E. coli*	- polymerase**	20,000
	E. coli	+ polymerase**	223,000
	B. subtilis	- polymerase**	43,000
	B. subtilis	+ polymerase**	244,000

* RNA produced by *in vitro* transcription of SP82 DNA by *B. subtilis* RNA polymerase as described by Hiatt and Whiteley (4).

** RNA polymerase isolated from rifampin-resistant *B. subtilis*, conditions for uncoupled and coupled translation described by Hiatt and Whiteley (4).

sent in the SDS eluates from each of the polymerase-Sepharose columns. Co-electrophoresis of the SDS eluates with the purified 28K peptide in a two-dimensional gel system revealed a labeled peptide which migrated to precisely the same position as the purified 28K peptide (data not shown). The experiments shown in Fig. 1 indicate that mRNA coding for the 28K peptide was synthesized *in vivo* by 4 min after infection, that synthesis of this mRNA did not require protein synthesis and that the *E. coli* cell-free preparation was capable of translating this mRNA. As seen from the pattern of bands in lanes A and D of Fig. 1, the 28K peptide was not easily detectable among the other translation products.

Synthesis of the 28K peptide by translation of RNAs produced in vitro. RNA synthesized *in vitro* by *B. subtilis* RNA polymerase from SP82 DNA was purified and translated with S-30 fractions from *E. coli* and *B. subtilis* ("uncoupled translation"). Experiments were also performed with coupled *B. subtilis* transcription - *E. coli* translation and with a coupled transcription-translation system derived from *B. subtilis*. Table 1 shows that all four systems were capable of incorporating ^{35}S-methionine into trichloracetic

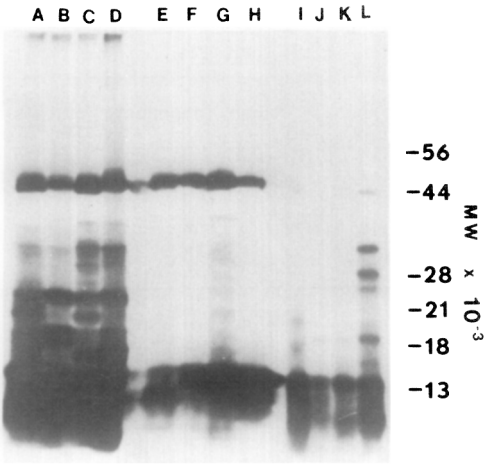

Figure 2. Autoradiogram resulting from the one-dimensional SDS-polyacrylamide gel electrophoresis of peptides synthesized in vitro from RNAs produced in vitro and subsequently adsorbed to columns of RNA polymerase-Sepharose.

Four reaction mixtures were tested: 1) an uncoupled E. coli translation system directed by RNA produced by B. subtilis RNA polymerase from SP82 DNA; 2) an uncoupled B. subtilis translation system directed by RNA produced by B. subtilis RNA polymerase from SP82 DNA; 3) a coupled system containing E. coli translation components supplemented with rifampin-resistant B. subtilis RNA polymerase and SP82 DNA as described earlier (4); 4) a coupled system containing B. subtilis translation components supplemented with rifampin-resistant B. subtilis RNA polymerase and SP82 DNA using conditions described earlier (4). The in vitro synthesized polypeptides used in this experiment were from the experiment shown in Table 1. Equal amounts of radioactivity (8 x 10^6 cpm) were adsorbed to RNA polymerase-Sepharose columns for each sample. Lanes A-D: total peptides produced by mixtures 1-4, respectively, prior to chromatography on columns of RNA polymerase-Sepharose (each lane contained 200,000 cpm); lanes E-H, total radioactive peptides eluted by 1.0 M NaCl after adsorption of the translation products synthesized by mixtures 1-4, respectively, to columns of RNA polymerase-Sepharose; lanes I-L total radioactive peptides eluted by SDS after adsorption of translation products synthesized by mixtures 1-4, respectively, to columns of RNA polymerase-Sepharose.

acid-precipitable material. The translation products from the experiments shown in Table 1 were analyzed as in the experiments illustrated in Fig. 1.

Synthesis of the 28K peptide was not detected in experiments using uncoupled transcription-translation when translation was provided by either an E. coli extract (lanes E and I of Fig. 2) or a B. subtilis extract (lanes F and J of Fig. 2). A similarly negative result was obtained in experiments with coupled B. subtilis transcription - E. coli translation (lanes G and K of Fig. 2). However, as seen in lane L of Fig. 2, one of the peptides synthesized by

the B. subtilis coupled transcription-translation system had the same electrophoretic mobility as the 28K peptide. Synthesis of this peptide was confirmed by analysis of the translation products in a two-dimensional gel system (data not shown). The identities of the other bands in lane L are not known. The peptide with a molecular weight of 35,000 may be analogous to a peptide of the same molecular weight synthesized from 4 min RNA (lane E, Fig. 1).

Discussion

The in vitro synthesis of the 28K peptide was detected only by coupled transcription-translation using a system derived from B. subtilis and not in experiments with uncoupled translation or in a B. subtilis transcription - E. coli translation system. In view of the observation (Fig. 1) that RNA extracted from SP82-infected cells directed the synthesis of the 28K peptide by an E. coli fraction, it seems unlikely that a specific B. subtilis translation factor is required for the synthesis of the 28K peptide.

Most of the RNA synthesized in vitro by the unmodified polymerase comes from transcription of genes having strong early SP82 promoters (2,6). Almost all of these genes are located on the terminally redundant portion of the genome (2,14) and are therefore present in two copies. These genes are transcribed in vivo very early in infection (during 1-3 min pulse-labeling interval, 6) and then transcription of some of these genes is shut off (3,15). The mechanism(s) involved in the in vivo shut-off are not known. Studies of complex formation (6) showed that restriction fragments containing strong early promoters formed polymerase-DNA complexes in the presence of 0.2 M NaCl and at low enzyme-DNA ratios. Genes with weak early promoters are not located in the terminally redundant regions and are transcribed in vivo at a slightly later time in infection (during the 3-5 min pulse-labeling interval, 6); restriction fragments with weak early promoters were not stably bound at 0.2 M NaCl and did not form complexes at low enzyme-DNA ratios. Possibly, the 28K peptide is encoded on a gene having a weak early promoter and transcription of such genes in

vitro is more efficient when coupled to translation. Presumably coupling with the *B. subtilis* translational system was more effective than coupling with the *E. coli* cell-free extract. It would be expected that *in vivo* transcription of such genes having weak early promoters would be enhanced not only by coupling with translation but also by the shut-off of transcription from some of the strong early promoters.

Acknowledgements

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References

1. Whiteley, H.R., Spiegelman, G.B., Lawrie, J.M. and Hiatt, W.R. (1976) RNA Polymerase (Losick, R., and Chamberlin, M., eds) pp. 587-600, Cold Spring Harbor, N.Y.
2. Lawrie, J.M., Spiegelman, G.B. and Whiteley, H.R. (1977) Gene 2, 251-262.
3. Rabussay, D. and Geiduschek, E.P. (1977) Comprehensive Virol. 8 (Fraenkel-Conrat, H. and Wagner, R.R., eds) pp. 1-196.
4. Hiatt, W.R. and Whiteley, H.R. (1978) J. Virol. 25, 616-619.
5. Spiegelman, G.B. and Whiteley, H.R. (1978) Biochem. Biophys. Res. Comm. 81, 1058-1965.
6. Achberger, E.C. and Whiteley, H.R. (1980) J. Biol. Chem. 255, 11957-11964.
7. Spiegelman, G.B., Hiatt, W.R. and Whiteley, H.R. (1978) J. Biol. Chem. 253, 1756-1765.
8. Hagen, F. and Young, E.T. (1973) Virol. 55, 231-241.
9. Millet, J. (1970) J. Appl. Bacteriol. 33, 207-219.
10. Haworth, S.R. and Brown, L.R. (1973) J. Bacteriol. 114, 103-113.
11. Ratner, D. (1974) J. Mol. Biol. 88, 373-383.
12. March, S.C., Parikh, I. and Cuatrecases, P. (1974) Anal. Biochem. 60, 149-152.
13. Panganiban, A.T. and Whiteley, H.R. (1981) J. Virol. 37, 373-382.
14. Lawrie, J.M., Spiegelman, G.B. and Whiteley, H.R. (1976) J. Virol. 19, 359-373.