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Conversion of *Bacillus subtilis* RNA Polymerase Activity *In Vitro* by a Protein Induced by Phage SP01

(RNA nucleotidyltransferase purification/*in vitro* modification/subunit binding/σ factor)

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ABSTRACT **A protein fraction from *B. subtilis* infected with phage SP01 (fraction LGG) stimulates the activity of RNA polymerase (EC 2.7.7.6; nucleosidetriphosphate:RNA nucleotidyltransferase) core from uninfected bacteria. Fraction LGG contains a protein (P^{28}, molecular weight 28,000) that is labeled after phage infection and binds tightly to RNA polymerase core at a relatively high ionic strength. *B. subtilis* RNA polymerase core with bound P^{28} has the transcription specificity of the previously purified, phage-modified B-P RNA polymerase; the latter contains two subunits, ν^{28} and ν^{13} (molecular weights 28,000 and 13,000, respectively) that are synthesized after phage infection. Both enzymes transcribe SP01 DNA preferentially and direct the asymmetric synthesis of viral middle RNA. P^{28}, like ν^{28}, binds more tightly to *B. subtilis* RNA polymerase core than the *B. subtilis* initiation factor, σ, at higher ionic strength. We propose that P^{28} and ν^{28} are the same protein. P^{28} and, by implication, ν^{28} suffice to endow the bacterial RNA polymerase core with a novel transcription specificity.**

RNA polymerase (EC 2.7.7.6; nucleosidetriphosphate:RNA nucleotidyltransferase) is a determinant of genetic regulation in the sense that its mechanism of action and its repertoire of interactions determine strategies of transcriptional control. Development of the large bacterial DNA viruses stems from programs of gene expression in which regulation is evidently largely at the transcriptional level. Bacterial host RNA polymerase is a target of this regulation through the binding of virus-coded peptides (1–3) or through modification (4–6). In previous experiments, we have shown that two peptides, ν^{28} and ν^{13}, that are synthesized after infection of *Bacillus subtilis* by phage SP01, bind tightly to bacterial RNA polymerase core. The resulting enzyme (called B-P), consisting of subunits ν^{28} (molecular weight 28,000), ν^{13} (molecular weight 13,000), and RNA polymerase core subunits β, β', α, ω^1, and ω^2 (the latter two newly identified subunits of molecular weight 11,000 and 9,500, respectively), selectively transcribes mature phage SP01 DNA to yield asymmetric viral middle RNA *in vitro* (7–9). In contrast, unmodified bacterial RNA polymerase holoenzyme (constitution β, β', σ, α, ω^1, ω^2) selectively transcribes only the SP01 early genes (8–10). Spiegelman and Whiteley (11) and Pero and collaborators (12, 13) have also found several new peptides bound to RNA polymerase after phage SP82 and SP01 infection and have noted altered transcription specificity. But are these peptides, either singly or together, the determinants of an altered transcription specificity? The experiments presented here suggest that peptide ν^{28} is such a determinant.

Abbreviation: LGG, light glycerol gradient fractions.

MATERIALS AND METHODS

Growth of *B. subtilis* 168M (indole$^-$), SP01 phage infection, [^{3}H]leucine labeling of bacteria, and SP01 DNA preparation are described elsewhere (9). ϕ1 DNA was a gift of J. Ito. Electrophoresis of 12.5% polyacrylamide gels in 0.1% (w/v) sodium dodecyl sulfate-containing buffer was according to Laemmli (14). Gel fixing, staining, destaining, scanning, slicing, and determination of ^{3}H have been described (9). Centrifugation through 4.5-ml linear 10–30% (v/v) glycerol gradients in buffer D (10 mM Tris·HCl pH 8, 1 mM EDTA, 0.1 mM dithiothreitol) was done with varying concentrations of KCl and, where noted, 0.01% Triton X-100 was present to improve recovery of protein. Samples of less than 0.5 ml were layered on top of these gradients and centrifuged at 60,000 rpm (358,000 $\times$ g_{max}, Beckman SW65 rotor) for 8.5 hr at 4°. Gradients were collected from the bottom, through a hypodermic needle. Hybridization-competition analysis and its analytical rationale have been described (8, 9). RNA·RNA duplex formation was assayed according to ref. 15. ^{3}H-Labeled proteins from glycerol gradients were spotted on glass fiber filters (Whatman GF/C), dried, digested with tissue solubilizer (Protosol, New England Nuclear, 1 hr, 60°), and counted in toluene-based scintillation fluid.

Assays for RNA polymerase activity were preceded by a 10-min incubation at 30° of the following components (volume 90 μl; assembled on ice): 10 μmol of Tris·HCl (pH 8.0), 0.01 μmol of EDTA, 0.08 μmol of spermidine·Cl, 0.01 μmol of dithiothreitol, 10 μg of bovine serum albumin, 0.1 μmol each of ATP, GTP, and CTP, 0.01 μmol of [^{3}H]UTP (6–12 cpm/pmol) or [α-^{32}P]UTP (150–300 cpm/pmol), 3–4 μg of DNA, and enzyme. RNA polymerization was started either by: (*i*) adding 10 μl of $MgCl_2$ to a final concentration of 10 mM, or (*ii*) simultaneously adding $MgCl_2$ and rifampicin in 10 μl to a final concentration of 10 mM and 5 μg/ml, respectively. The latter method of assaying limits activity to RNA polymerase molecules that have formed tightly binding, rapidly initiating complexes with DNA during the preincubation (16). We call this the rapid start or "RS" assay. When assaying for the ability to stimulate RNA polymerase core, the material in question was added to all the other components of the above preincubation mixture on ice. Preincubation was initiated by adding RNA polymerase core followed by transfer to 30°. RNA synthesis was terminated and samples were prepared for determination of radioactivity as described (8, 9).

Purification of RNA polymerase from large quantities of cells (greater than 100 g wet weight) has been described elsewhere (procedure 1, ref. 9). A modification of that pro-

cedure for use with small quantities of cells (not more than 10 g) is described here (procedure 2). All buffers are described in ref. 9.

Step 1: Frozen cells (5–10 g wet weight) were thawed in 15–30 ml of buffer BCMG containing protease inhibitors, passed through a French press at 10,000–12,000 lb/inch^2 (70,000–84,000 kPa) and centrifuged at 20,000 × g for 30 min. The supernatant was collected (Fraction I).

Step 2: Fraction I was brought to 25% saturation with solid $(NH_4)_2SO_4$ (14.4 g/100 ml) and adjusted to pH 8 with 5 M NH_4OH. After the fraction was stirred on ice for 30 min, it was centrifuged at 314,000 × g_{max} for 1.5 hr (supernatant = Fraction II).

Step 3: Fraction II was 70% saturated with solid $(NH_4)_2SO_4$ (30.7 g/100 ml of Fraction II) and pH was adjusted as above. The mixture was stirred on ice for 30 min, sonicated (9), and centrifuged at 34,000 × g_{max} for 30 min. The pellet was dissolved in a minimum volume of buffer B containing 50 mM KCl (Fraction III).

Step 4: Fraction III was rapidly dialyzed (30 min) twice against 1 liter of buffer B with 50 mM KCl and 0.1% Triton X-100, and applied to a DEAE-cellulose column (2.5 cm inner diameter × 8 cm, equilibrated with the same buffer) at 40 ml/hr. The column was washed with 50 ml of the same buffer, and enzyme activity was eluted with 0.35 M KCl, 0.1% Triton X-100 in buffer B. Fractions with polymerase activity were pooled (Fraction IV).

Step 5: Fraction IV was diluted to 0.15 M KCl with buffer B, applied to a calf thymus DNA-cellulose column (2.5 cm inner diameter × 8 cm, equilibrated with 0.15 M KCl in buffer B) at 60 ml/hr, and washed with 100 ml of the same buffer. A linear gradient (300 ml) of 0.15–1.0 M KCl in buffer B was applied. Elution patterns of RNA polymerase activity followed those seen with purification procedure 1 (9). RNA polymerase fractions corresponding to the previously described VA, VB, and VC fractions were separatedly pooled, concentrated by $(NH_4)_2SO_4$ precipitation, dissolved in buffer D containing 5% glycerol (v/v) and 0.5 M KCl, and used immediately in the next step.

Step 6: Fractions VA, VB, and VC were layered onto separate 30-ml 10–30% (v/v) glycerol gradients in buffer D with 0.5 M KCl and centrifuged at 90,000 × g_{max} (25,000 rpm, Beckman rotor SW25) at 4° for 48 hr. Fractions were collected, assayed for activity, pooled, precipitated with $(NH_4)_2SO_4$, dissolved in buffer C, and stored at −20°.

RESULTS

A SP01 DNA-Specific Transcription Factor. Our first experiment concerns *B. subtilis* that have been labeled with [^{3}H]leucine 2.5–10 min after infection at 37° with phage SP01 and have been collected at 10 min of the infection. When RNA polymerase was purified from these cells by procedure 2 of *Materials and Methods,* the last step of the purification of fraction VB, glycerol gradient centrifugation in 0.5 M KCl, separated RNA polymerase activity from those slowly sedimenting, ^{3}H-labeled proteins that were confined to the upper 25% of the gradient. The pooled fractions (designated LGG for *l*ight *g*lycerol *g*radient) were concentrated by $(NH_4)_2SO_4$ precipitation and analyzed on polyacrylamide gels. Fig. 1 shows fraction LGG to contain many unlabeled bacterial proteins and six major bands of ^{3}H-labeled (presumably phage-induced) proteins ranging in molecular weight from 125,000 to 13,000. They include an unlabeled protein banding at the position of σ factor and labeled proteins, P28 and P13, banding at the positions of ν^{28} and ν^{13}. Needless to say, their mobilities do not unequivocally identify P^{28} and P^{13} as ν^{28} and ν^{13}. In three preparations of unlabeled LGG, the pattern of protein bands remained similar with only the relative proportions varying; in one preparation of ^{3}H-labeled LGG, the 38,000 molecular weight protein comprised 90% of the label, with the 28,000 molecular weight protein making up the remainder.

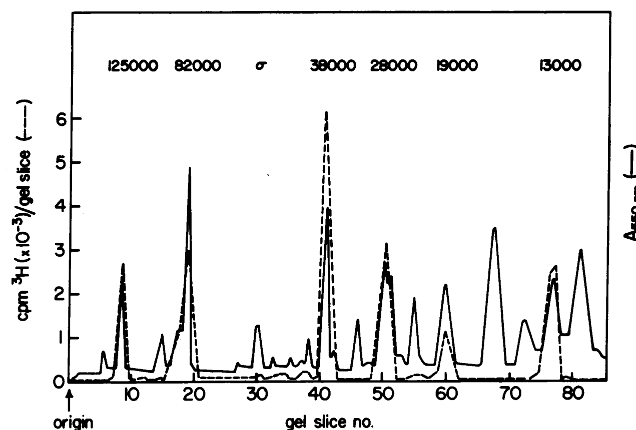

FIG. 1. Densitometric profile and [^{3}H]leucine incorporation of proteins present in fraction LGG. The top 25% of a glycerol gradient used in the purification of RNA polymerase by procedure 2 (*Materials and Methods*) was concentrated by ammonium sulfate precipitation. An aliquot was electrophoresed on a 12.5% polyacrylamide gel, stained, destained, scanned, sliced, and counted. Stain density (——); [^{3}H]leucine incorporation (– – –). Molecular weights of the major ^{3}H-labeled components and the position of σ factor are shown at the top of the graph.

RNA polymerase core from uninfected *B. subtilis* is stimulated by LGG (Table 1, line 2). Stimulation is preferentially for SP01 DNA relative to φ1 DNA. Preference for SP01 DNA is also a property of the phage B-P RNA polymerase (Table 1, line 4) but not of *B. subtilis* RNA holopolymerase

TABLE 1. *Ability of RNA polymerase to form rapidly initiating complexes with SP01 and φ1 DNA*

Enzyme	Template	pmol UMP incorporated/ μg of enzyme	Activity ratio φ1/SP01
(*1*) *B. subtilis* core	SP01	1.5*	1.3
	φ1	1.9	
(*2*) *B. subtilis* core + LGG	SP01	19.8*	0.29
	φ1	5.7	
(*3*) *B. subtilis* holoenzyme	SP01	12.5†	1.1
	φ1	13.7	
(*4*) B-P polymerase	SP01	15.5†	<0.05‡
	φ1	<1	

* RS assays, described in *Materials and Methods,* contained 1.5 μg/ml of RNA polymerase core, 20 μg/ml of SP01 or φ1 DNA, and [α-^{32}P]UTP (specific activity, 180 cpm/pmol).

† RS assays contained 5 μg/ml of holoenzyme or 10 μg/ml of B-P polymerase, 20 μg/ml of SP01 or φ1 DNA, and [^{3}H]UTP (specific activity, 9.8 cpm/pmol).

‡ Values ranged from <0.03 to 0.17 for different B-P polymerase preparations and assays, and averaged 0.10.

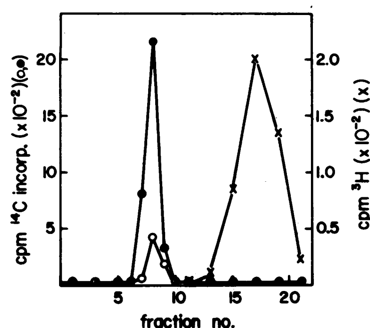

FIG. 2. Glycerol gradient centrifugation of RNA polymerase with and without fraction LGG. Two hundred microliters of dialyzed [buffer D with 0.5 M KCl and 5% (v/v) glycerol] ^{3}H-labeled fraction LGG was combined with 50 μg of *B. subtilis* RNA polymerase core, incubated for 5 min at 30°, and centrifuged through 10–30% glycerol in buffer D with 0.5 M KCl. A comparison gradient contained 50 μg of RNA polymerase core in 200 μl of dialysis buffer, treated as above. Aliquots (10 μl each) were assayed for RNA polymerase activity (●, core +LGG; O, core alone) or for ^{3}H-labeled protein (X, aliquots were too small to detect ^{3}H in fractions 1–10). Sedimentation is from right to left.

or core (Table 1, lines 1 and 3). However, LGG does stimulate RNA polymerase core activity on φ1 DNA while B-P polymerase does not have a greater activity on φ1 DNA than polymerase core (Table 1, lines 2 and 4). We conclude that part of the stimulating activity of LGG may be due to host initiation factor, σ. However, LGG must also contain a stimulating factor that is specific for SP01 DNA since it is not possible to derive the result on line 2 of Table 1 from any linear combination of lines 1 and 3.

Conversion of RNA Polymerase Core In Vitro. When *B. subtilis* RNA polymerase core was combined with ^{3}H-labeled LGG and then centrifuged through a glycerol gradient, a strikingly increased recovery of RNA polymerase activity was observed, relative to a companion gradient containing only RNA polymerase core (Fig. 2). The 5-fold increase in polymerase activity was specific to SP01 DNA as template;

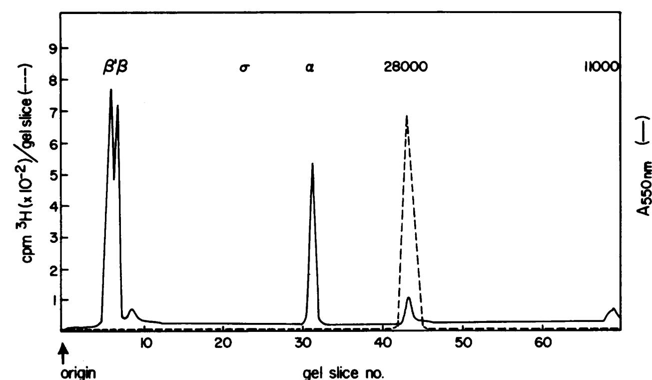

FIG. 3. Densitometric and ^{3}H profile of RNA polymerase core after incubation with fraction LGG and glycerol gradient centrifugation. Fractions 7 and 8 from Fig. 2 were combined, electrophoresed, stained, destained, scanned, sliced, and counted as in Fig. 1. Stain density (——); radioactivity (– – –). Subunit designations and molecular weights are shown at the top of the graph.

activity of the centrifuged RNA polymerase on φ1 DNA had not been stimulated by LGG. In four experiments of this kind the stimulation of SP01 DNA-directed activity of RNA polymerase due to fraction LGG (assayed after glycerol gradient centrifugation) averaged 5.5-fold (range 4.6- to 6.6-fold).

The subunit composition of LGG-stimulated RNA polymerase core was examined. Peak fractions of enzyme activity (Fig. 2) were pooled and electrophoresed (Fig. 3). Only one ^{3}H-labeled peptide, P^{28}, bound to the enzyme. Stained gels showed the β, β′, α, and ω^1 subunits of the core as previously reported (ref. 9; ω^2 was not resolved on these gels) and the peptide P28 with the mobility of ν^{28}. In three experiments the molar proportions of $P^{28}:\alpha$ ranged from 0.5 to 0.9:2. In two separate experiments, minor proportions of other peptides were also found—an unlabeled peptide of molecular weight 110,000 in one instance ($P^{110}:\alpha = 0.12:2$) and an unlabeled peptide of molecular weight 75,000 ($P^{75}:\alpha = 0.16:2$) in the other instance. The SP01-specific phage B-P polymerase contains ν^{13} as well as ν^{28}, and fraction LGG contains a ^{3}H-labeled peptide, P^{13}, with the mobility of ν^{13} (Fig. 1), yet this peptide never bound to RNA polymerase (limits of detection $P^{13}:\alpha = <0.05:2$). While we do not know that P^{13} and ν^{13} are the same, we can conclude that the template-selective properties of gradient-purified, LGG-stimulated *B. subtilis* RNA polymerase core do not depend on ν^{13}. The next experiment analyzes the SP01 RNA synthesized by this enzyme (Fig. 4) and compares it with SP01 RNA made with phage B-P polymerase (8, 9). The similarity of the two RNA samples is evident. Both show selective synthesis of viral middle RNA (detailed arguments for this conclusion from the data are given in refs. 8 and 9). The substantially different competition of *in vivo* 28-min RNA suggests that the two RNA preparations contain different proportions of the m_2l (8, 9) subclass of middle transcripts. We have not yet pursued the interesting implications of this result.

Symmetry of the two RNA samples (Table 2) is low and also entirely comparable. Comparison of lines 1, 2, and 4 of Table 2 shows that the middle RNA of both samples, which accounts for approximately 60% of the total, *is entirely asymmetric*. In contrast, *B. subtilis* RNA polymerase core yields symmetric SP01 RNA transcripts of the early, middle, and late genes (9).

From these experiments, we surmise that P^{28} of fraction LGG is equivalent to ν^{28} of B-P RNA polymerase from phage-infected *B. subtilis*. Of all the bacterial and phage-induced proteins present in fraction LGG, only P^{28}/ν^{28} binds in repeated experiments to *B. subtilis* RNA polymerase core, under the conditions of Fig. 2, and alone converts RNA polymerase core from uninfected cells to an enzyme having the properties of the phage B-P polymerase.

Influence of Salt Concentration on Binding of σ and P^{28}/ν^{28} to RNA Polymerase Core. When RNA polymerase core and fraction LGG were combined under conditions described in the legend of Fig. 2 but at lower ionic strength (0.1 M KCl) and separated on a glycerol gradient also containing 0.1 M KCl, the properties of the recovered polymerase differed from those described in the previous section. The LGG-specific stimulation could be assayed on SP01 DNA (3.6-fold increase relative to control; see Fig. 2), but also on φ1 DNA (3.1-fold increase). The pooled peak fractions were able to form RS complexes with φ1 DNA and SP01 DNA (data not shown).

A densitometric scan of a gel electropherogram of the pooled polymerase peak now showed, in addition to β', β, α, and P^{28}, a protein with the mobility of σ (P^{55}, molecular weight 55,000). The molar ratio α:P^{28}:P^{55} was 2:0.9:0.8.

The SP01 RNA made in the presence of this polymerase differed from that described in Fig. 4 and resembled RNA made by B-P polymerase in the presence of saturating amounts of σ factor (ref. 9, Fig. 11b): it contained less middle and more early RNA, including *e* RNA, and was asymmetric (data not shown). These properties indicate that the P^{55} of fraction LGG is indeed σ, but that σ can bind to polymerase core only at the lower salt concentration of this last experiment. This conclusion was tested by glycerol gradient centrifugation of holoenzyme in buffer D containing varying concentrations of KCl. Loss of σ factor was tested in three ways: by recovery of polymerase activity, by ability of purified σ to stimulate the activity of the polymerase recovered after centrifugation, and by measurement of σ activity in the supernatant portions of the glycerol gradient. σ Factor was found to be released during centrifugation at greater than 0.3 M KCl, with partial retention at 0.2 M KCl, and no significant loss at 0.1 M KCl or less. This is in contrast to P^{28}/ν^{28} which, as shown in previous sections, can bind to polymerase core at 0.5 M KCl. ν^{28} is at least partly retained by enzyme B-P after glycerol gradient centrifugation at 1.0 M KCl (data not shown). Thus, P^{28}/ν^{28} binds to RNA polymerase core significantly more strongly than σ factor.

DISCUSSION

The primary aim of these experiments has been to demonstrate the conversion of transcription specificity of RNA polymerase from uninfected *B. subtilis in vitro*. We have provided the material for this conversion from a fraction that cochromatographs with RNA polymerase on DNA-cellulose. This fraction contains an RNA polymerase-binding protein, P^{28}, which appears to be an unbound form of the previously identified subunit, ν^{28}, of phage-modified RNA polymerase B-P (9). The conversion, which only requires the binding of phage-induced P^{28}, redirects the transcription properties of RNA polymerase core so that highly asymmetric middle SP01 RNA (Fig. 4 and Table 2) rather than symmetric RNA (9) is synthesized. SP01 RNA made by P^{28}-converted core polymerase and by the B-P polymerase (9) contains similar proportions of viral middle transcripts. We surmise that P^{28} and ν^{28} are identical and that the ν^{28} subunit is a specificity determinant for phage SP01 middle RNA synthesis. An additional role for the ν^{13} subunit in SP01 middle RNA synthesis is by no means excluded. For example, Fig. 4 suggests some difference in the *in vitro* synthesis of the m_2l subclass of middle SP01 transcripts with the *in vitro*-converted and the B-P RNA polymerases. We do not know whether this difference reflects the presence or absence of ν^{13} subunit in RNA polymerase. Detailed analyses of the different subclasses of viral middle RNA that are synthesized *in vitro* by various enzymes remain to be carried out.

Recently, Pero *et al.* (13) reported the isolation of an RNA polymerase fraction (designated as A) from phage SP01-infected *B. subtilis* that synthesizes SP01 RNA, including viral middle transcripts, with considerable asymmetry. This polymerase fraction contains a host peptide (designated as δ) to which Pero and coworkers assign an asymmetry-determining role for viral middle transcription. However, it has already been shown that enzyme B-P synthesizes viral middle RNA with almost perfect asymmetry (8), and we have shown here that P^{28}/ν^{28} addition to *B. subtilis* RNA polymerase core suffices to direct this asymmetric transcription. The relevant evidence (Table 2) comes from measurements of RNA·RNA

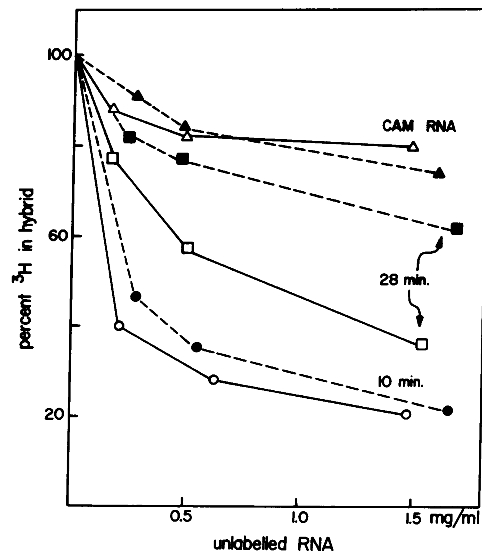

FIG. 4. Hybridization-competition of SP01 [³H]RNA synthesized *in vitro* with *B. subtilis* RNA polymerase core with bound P^{28} from a peak fraction after centrifugation (as Fig. 3). RNA was synthesized at DNA excess for 2 min at 30°, with initiation of RNA chains confined to the first 30 sec of synthesis by addition of rifampicin, as described (9). Hybridization of [³H]RNA (0.02 µg/ml) to SP01 DNA (10 µg/ml) was competed by unlabeled RNA from SP01-infected *B. subtilis:* Δ, CAM early RNA; O, 10-min middle RNA; □, 28-min late RNA. Input radioactivity was 908 cpm per assay; 650 cpm of RNA hybridized to DNA in the absence of unlabeled competitor RNA; the background of radioactivity bound to the filters in the absence of DNA was 3 cpm. The dotted lines (*filled symbols*) allow a comparison to be made with RNA synthesized under similar conditions with phage B-P RNA polymerase (ref. 9, Fig. 9a).

TABLE 2. *Asymmetry of SP01 RNA synthesized by B. subtilis RNA polymerase core with bound P^{28}/ν^{28}*

Unlabeled RNA *in vivo*	% [³H]RNA in duplex*
None	1 (5)†
(*1*) CAM (early)	14 (16)
(*2*) 10 min (middle)	10 (13)
(*3*) 28 min (late)	13 (7)
(*4*) CAM + 10 min	11 (14)

* The [³H]RNA used for hybridization-competition (Fig. 4) was also analyzed for formation of RNA·RNA duplexes with *in vivo* RNA from phage SP01-infected *B. subtilis*. Each sample, containing 0.02 µg/ml (908 cpm) of [³H]RNA and, where indicated, 1.5–1.6 mg/ml of each *in vivo*, unlabeled RNA, was annealed and analyzed as described (15). A control sample was heated to 100° in the absence of added salt, cooled, and digested. The residual trichloroacetic acid-precipitable radioactivity (30 cpm) was subtracted from the above values as background.

† Values in parentheses refer to the same analysis for a comparable RNA sample made with B-P RNA polymerase isolated from phage-infected bacteria and already presented in ref. 9, Table VI.

duplex formation (17) with mixtures of *in vivo* RNA (18), which permits us to assign anti-messenger species to their conjugate classes of complementary *in vivo* messages. The anti-sense RNA that is produced *in vitro* in these experiments evidently is complementary to viral early (*em*) transcripts or to transcripts that are present in all RNA *in vivo*. In order to reconcile our prior results with the subsequent experiments of Pero and coworkers, we suppose that δ might suppress the synthesis of anti-early RNA by an enzyme that otherwise may resemble the P^{28}/ν^{28}-converted B-P polymerase in composition if not in stoichiometry.

We have used the RNA polymerase-binding property of P^{28}/ν^{28} to purify it from other phage and host proteins (Figs. 1 and 3). However, P^{28}/ν^{28} may also be a DNA-binding protein since it coelutes with the RNA polymerase at moderately high salt from DNA-cellulose. Its DNA-binding specificity remains to be investigated. Fraction LGG also contains several peptides that are labeled after infection. However, molecular weight coincidence alone is not a strong criterion of identity for comparison with virus-coded proteins that bind to RNA polymerase (9, 12, 13). Since these peptides in LGG do not bind to RNA polymerase core under our conditions, we cannot comment further on them. Fraction LGG also contains σ; however, it seems likely that σ merely dissociates from RNA polymerase during the glycerol gradient centrifugation in 0.5 M KCl that generates fraction LGG. The ability of P^{28}/ν^{28} to bind more strongly to RNA polymerase than σ factor implies possible control mechanisms *in vivo* that might regulate the competition between σ- and ν^{28}-mediated SP01 RNA synthesis.

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