AZOTOBACTER VINELANDII RNA POLYMERASE VI. AN IN SITU ASSAY FOR POLYMERASE AFTER ACRYLAMIDE GEL ELECTROPHORESIS.*

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Analysis of the A. vinelandii RNA polymerase by acrylamide gel electrophoresis indicated that enzyme activity was associated with more than one of the resolved protein bands. Initially RNA polymerase activity was determined after elution from gels sliced into discs (Chrambach, 1966). However, recovery of enzyme activity was variable and closely spaced protein bands could not be consistently assayed separately. The present paper describes a procedure for assaying for RNA polymerase in the acrylamide gel. This assay utilizes the unprimed synthesis of poly A·poly U (Smith et al, 1967; Krakow, 1968) or the rIC copolymer (Krakow and Karstadt, 1967) followed by staining with ethidium bromide to visualize the polymers.

EXPERIMENTAL PROCEDURES

The A. vinelandii RNA polymerase was prepared by a modification of the published procedure (Krakow and Ochoa, 1963) with gel filtration

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on agarose beads (Bio-Gel A - 1.5 m, BioRad Laboratory) replacing the final hydroxylapatite step. The RNA polymerase used in this paper had a specific activity of 150 units/mg (1 unit = 1 mμmole of C^{14}-AMP incorporated into RNA per min at 37° with calf thymus DNA as the template).

The enzyme was resolved by electrophoresis on 5% acrylamide gels using the Tris-Glycine buffer system described in the Canalco manual. The gels were polymerized with ammonium persulfate and the upper electrophoresis buffer contained 0.1 ml of 95% thioglycolic acid (Pierce Chemical Co.) per liter to avoid possible persulfate produced artifacts (Brewer, 1967). The Buchler polyanalyst electrophoresis apparatus was used with water circulating through the jacket at 5°. The enzyme was applied in .05 ml of .02 M Tris, pH 7.8-10% sucrose and electrophoresis was performed at 2.5 mamp per tube for 70 min. Protein was stained with aniline black and destained electrophoretically.

In situ assay for RNA polymerase: After electrophoresis, the gels were removed from the glass columns and placed in test tubes (13 x 100 mm) containing 5 ml of the following reaction mixture: 0.05M Tris, pH 7.8; 0.02M mercaptoethylamine, 2 mM $MnSO_4$; 0.6 mM ATP and 0.6 mM UTP for poly A·poly U synthesis. For the rIC copolymer reaction, 0.6 mM ITP and 0.6 mM CTP replaced ATP and UTP. The reactions were allowed to incubate overnight (18 hrs.) at 37°. The incubated gels were rinsed with 5 ml of 0.01 M Tris, pH 7.8 - 0.05 M NaCl - 0.001 M EDTA (TNE) and stained with ethidium bromide (100 μg/ml of TNE buffer) at room temperature. After 24 hrs. unbound dye is removed (if desired for photographing gels) by placing the gels in 50 ml of the TNE buffer. The ribopolymer - ethidium bromide complex stains bright red against a clear background.

RESULTS

The unprimed synthesis of poly A·poly U or rIC copolymer by RNA polymerase in acrylamide gels showed requirements similar to those of reactions carried out in solution (Krakow and Karstadt, 1967; Krakow, 1968). The formation of ethidium bromide staining material required Mn^{++} and the complementary pairs of substrates (i.e., ITP + CTP or ATP + UTP) must be present to support ribopolymer synthesis (Figure 1). Characteristic of the unprimed synthesis of poly A·poly U or rIC copolymer in solution is the

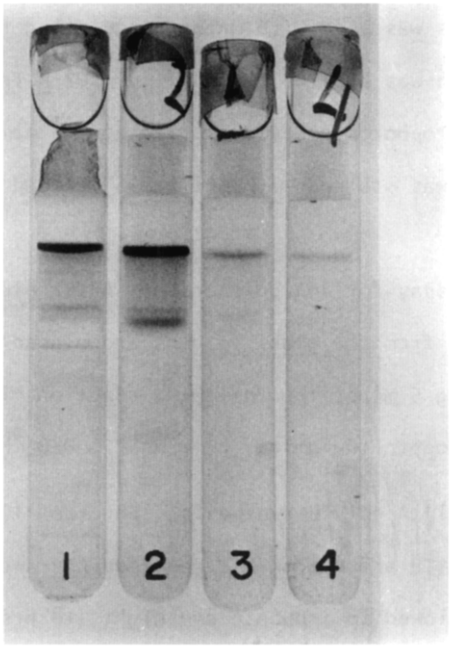

Figure 1. Assay for RNA polymerase in acrylamide gels. 10 μg of A. vinelandii RNA polymerase were applied to the gels and electrophoresed at 2.5 mamp per gel for 70 minutes. Gel 1 was stained for protein. Gel 2 was assayed for poly A·poly U synthesis; Gel 3, Mn^{++} omitted from assay mix; gel 4, ATP and UTP omitted from assay mix. The gels were incubated for 16 hours at 37° and stained with ethidium bromide as indicated in the text.

onset of turbidity in the reaction. When polymerase was assayed in the acrylamide gels, regions of high enzyme activity also showed a white precipitate in the region which subsequently stained red with ethidium bromide.

The onset of detectable (i.e., ethidium bromide stained polymer) poly A·poly U synthesis in the acrylamide gel is later than observed in soluble systems. The lag phase for poly A·poly U synthesis in the gel is about 30 minutes, in solution about 15 minutes at 37°. This is presumably due to the time required for the diffusion of the components of the reaction into the gel as

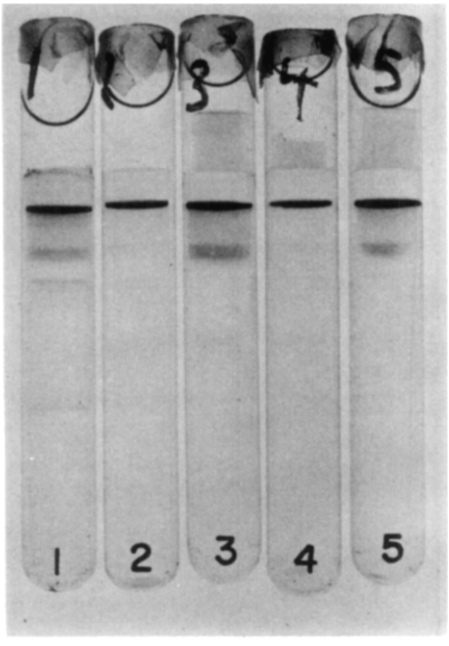

Figure 2. Effect of incubation time on polymerase activity in gels. 10 μg of RNA polymerase were resolved as in Figure 1. Gel 1 was stained for protein. Gels 2 and 3 were assayed for poly A·poly U synthesis; gel 2 was incubated for 1 hour at 37°, gel 3 for 5 hours at 37°. Gels 5 and 6 were assayed for rIC copolymer synthesis; gel 4 incubated for 1 hour at 37°; gel 5 for 5 hours at 37°. Gels 2-5 were stained with ethidium bromide.

well as the difference in the pH of the gel buffer which is above the optimum for the unprimed reaction. The major protein band shows the earliest appearance of polymer (Figure 2) and the minor regions of RNA polymerase activity show up best after 5 hr or overnight incubation. The lower limit of the *in situ* assay is about 1 μg of protein applied to the acrylamide gel with excellent results obtained at 5 μg of protein (Figure 3).

As can be seen from a comparison of the protein stained pattern and the polymerase assay there is a major, slow moving, band of protein and enzyme activity. This is contiguous with a

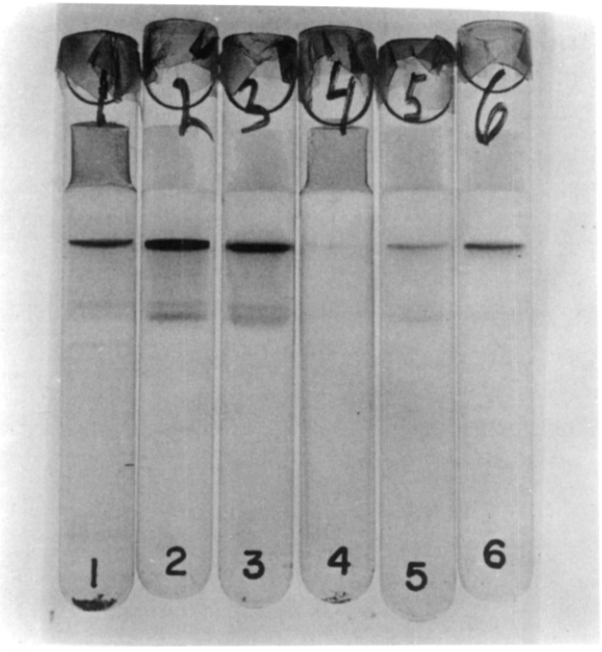

Figure 3. Sensitivity of the *in situ* polymerase assay. Gels 1-3 contain 5 μg and gels 4-6, 1 μg of RNA polymerase. The gels were run as given in Figure 1. Gels 1 and 4 were stained for protein. Gels 2 and 5 were assayed for poly A·poly U synthesis and gels 3 and 6 were assayed for rIC copolymer synthesis. The assays were incubated for 16 hours at 37° and stained with ethidium bromide.

diffuse area of activity which is bounded by a minor split band of protein and polymerase activity. The major band probably corresponds to the RNA polymerase dimer, the form found in low ionic strength. The minor bands correspond to monomeric forms of the enzyme and the diffuse area in the gel probably reflects a continued dissociation of the dimer. Incubation of polymerase with heparin (Figure 4) prior to electrophoresis resulted in a disappearance of the major band and all of the protein moved into the region of the minor bands. This was consonant with the results of Walter et al (1967) who showed that treatment of 24S E. coli RNA polymerase particles with heparin caused a quantitative transformation into material sedimenting around 15S.

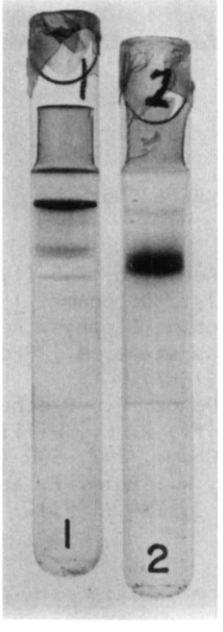

Figure 4. Dissociation of RNA polymerase dimer form by heparin. Gel 1 contained 15 μg of RNA polymerase, gel 2 contained 15 μg of RNA polymerase plus 5 μg of heparin. The gels were electrophoresed as in Figure 1 and stained for protein.

DISCUSSION

The assay described has the advantage of ease of operation and preserves the fine detail obtained after acrylamide disc electrophoresis. The stained protein patterns are closely matched by the patterns obtained after staining the polymers synthesized *in situ*. It is interesting to note that there was no difference in the acrylamide pattern noted for poly A·poly U or rIC copolymer synthesis, demonstrating that there is no apparent difference in the RNA polymerase responsible for the two types of unprimed reactions.

The only, and minor, problem encountered with the assay is the small amount of ethidium bromide, a cationic dye, which binds to protein in the absence of polymer synthesis (Figure 1). However, the ethidium bromide-protein complex stains a dull buff red and is difficult to mistake for the bright red dye-ribopolymer complex.

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