Poliovirus RNA replication requires the activities of a viral RNA-dependent RNA polymerase, 3D\textsubscript{pol}, in conjunction with several additional viral and likely cellular proteins. The importance of both the 3A and 3B coding regions has been documented previously by genetic tests, and their biochemical activities have been the subject of several recent investigations. In this study, we examined the previously reported stimulation of 3D\textsubscript{pol} RNA synthesis by 3AB. We show that 3AB does not stimulate RNA synthesis on templates that are stably base paired to a primer, indicating that 3AB does not stabilize or otherwise activate 3D\textsubscript{pol} for chain elongation. Similarly, it does not alter the kinetic parameters or binding affinities of 3D for substrates. In the absence of a primer, or in the presence of a primer that does not form a stable hybrid with the template, 3AB increases the utilization of 3'-hydroxyl termini as sites for chain elongation by 3D, and thereby stimulates RNA synthesis. 3AB may interact with and stabilize these sites and/or may recruit 3D\textsubscript{pol} to the site, resulting in stimulation of the initiation of elongation events. We propose that this activity is required for stabilizing weak interactions that occur during nucleotidyl-protein-primed initiation events in the viral RNA replication complex.

Poliovirus-infected cells contain an RNA-dependent RNA polymerase activity that has been attributed to a virally encoded protein, now known as 3D\textsubscript{pol} (1–3). A great deal of work over the past 25 years from many laboratories has been devoted to the purification and characterization of this enzyme, and several significant characteristics of 3D\textsubscript{pol} and its biological activities have been determined. The poliovirus RNA polymerase is a 52-kDa protein that has been purified in large quantities to yield a protein with very few, if any, detectable contaminants (4–6). Detailed x-ray structural studies of a major portion of the protein have been completed (7) and establish the basic features common to all types of nucleic acid polymerases (DNA-directed as well as RNA-directed); these include “fingers,” “palm,” and “thumb” subdomains (8, 9). The enzyme is dependent on both a template and a primer for activity and is responsible for RNA chain elongation during poliovirus RNA replication in infected cells. Although the natural primer for RNA synthesis in poliovirus-infected cells remains to be definitively identified, synthetic primers are commonly used in the laboratory for assay and characterization of 3D\textsubscript{pol} activity. It was proposed some years ago that structural elements in the RNA template may provide a mechanism for self-priming (10); however, a more current model involves protein priming (11). The enzyme has no apparent template specificity by itself, and yet it is known that in an infected cell 3D\textsubscript{pol} replicates only viral RNA.

The genome-linked protein, VPg (3B), plays an essential role in viral RNA replication, as mutations in the 3B coding sequence may abrogate RNA synthesis (12). It is found on the 5'-ends of all nascent RNA strands, including those not yet completed (13–15), indicating that attachment occurs at the time of or shortly after initiation of RNA synthesis. These observations led to the suggestion that VPg may serve as primer for initiation of RNA strand synthesis. Several polypeptides containing VPg sequences, including 3AB, have been detected in infected cells by immunoprecipitation with anti-VPg antibodies (16–18). These polypeptides are enriched in membrane fractions containing the viral RNA replication complexes isolated from infected cells. Using a partially purified 3D\textsubscript{pol} preparation, a “host factor,” and a poliovirion RNA template, product RNA was digested with RNase, and residual material was immunoprecipitated with anti-VPg serum; polyacrylamide gel electrophoretic analysis of this material showed 49 and 14 kDa proteins, the latter presumably 3AB, associated with residual RNA (19). This important result suggested that 3D and other proteins resident in this 3D preparation were sufficient to catalyze the transfer of 3AB to nascent RNA. Mutation of 3A generated a temperature-sensitive virus characterized by decreased synthesis of viral minus and/or plus strands at a non-permissive temperature (20, 21). It remains unclear, however, whether VPg (3B), 3AB, or some other precursor form serves as the donor of VPg to nascent RNA strands.

Previous reports have shown that 3AB stimulated 3D activity 50–100-fold in a poly(A)-oligo(dT) template-primer system (22, 23). With a heteropolymeric template and primer there was also substantial stimulation (approximately 10-fold) of product formed in the presence of 3AB. For unexplained reasons, the products were of varied sizes, both in the presence or absence of 3AB, including some quite large products greater than dimer length. With a poliovirion RNA template primed with oligo(dT), stimulation of 3D-catalyzed product formation by 3AB was 5–10-fold. In a different study, Plotch and Palant (24) used poliovirus minus strands in a primer-independent reaction with a high concentration of 3D; they reported that they observed about 75-fold stimulation of 3D activity by 3AB with no increase in the rate of chain elongation.

In addition to measurements of 3AB effects on template-dependent RNA synthesis by 3D\textsubscript{pol}, other reported 3AB activities include: (1) 3AB binds to 3CD and 3D (24, 25). (2) 3AB assists 3CD in forming a complex with the cloverleaf structure at the 5'-end of poliovirus plus strands and at the 3'-end of plus strands (26). (3) 3AB stimulates cleavage of 3CD to form 3C plus 3D (25). (4) 3AB binds to RNA in a sequence nonspecific

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manner (27). Interestingly a mutation, K107E, in 3AB decreased RNA binding and RNA plus and minus strand synthesis in mutant virus-infected cells. (5) 3AB associates with membranes as an integral membrane protein during in vitro translation (28).

It is not known whether any or all of these activities observed in vitro reflect significant contributions to the RNA replication reaction in an infected cell. Indeed, a biochemical mechanism for protein priming of RNA strand elongation has not been defined. Several laboratories have reported the formation of uridylylated forms of 3B (VPg), both in vivo and in vitro, that could form weakly base paired hybrids with the 3' terminal nucleotides of poliovirus plus and minus strand RNAs; and it is assumed that these nucleotidyl-proteins serve as primers for RNA synthesis. In this report we show that 3AB does not stimulate 3D-catalyzed reactions on templates that are stably base paired to a primer. In the absence of primer (or in the presence of a primer that does not form a stable hybrid with the template), 3AB allows more efficient utilization of 3'-hydroxyl termini that transiently form duplex regions. 3AB may interact with and stabilize these sites and/or may recruit the 3D polymerase to the site, resulting in stimulation of the initiation of elongation events. We suggest that this activity of 3AB allows initiation to occur on templates formed by weak interactions during nucleotidyl-protein-primed initiation events.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were obtained from Boehringer Mannheim, Life Technologies, Inc., or New England Biolabs, Inc. The expression vectors, pGEM-4Z and pGEM-3Z, and RNAins were obtained from Promega. Calf intestinal alkaline phosphatase, proteinase K, and calf thymus DNA were obtained from Sigma. DNA primers were synthesized by Genemed Biotechnologies, Inc. Escherichia coli JM110 was provided by Bert Semler, University of California, Irvine. E. coli BL21(DE3) was a gift from Steve Schultz, University of Colorado, and E. coli BL21(DE3)-pET7lac was a gift from Aniko Paul and Eckard Wimmer, State University of New York, Stony Brook. The in vitro transcription kits, T7 MEGAscript and SP6 MEGAscript, were purchased from Ambion, Inc. [β-32P]UTP (NET-380), [α-32P]UTP (BLU506H), and [α-32P]UTP (BLU507H) were obtained from NEW Life Sciences Products.

Construction of Expression Vectors—Poliovirus genome sequences 1–72 were inserted into the expression vector, pGEM-4Z, which was modified to remove sequences between the Smal and HindIII sites in the multiple cloning site. The vector was then digested with EcoRI and Asp718 and dephosphorylated with calf intestinal alkaline phosphatase and the large fragment was agarose gel purified and isolated with the Qiaex II extraction kit (Qiaex, Inc.). The plasmid pGEM-4Z-PV1-72 was digested with HindIII and Asp718, and extracted DNA was transcribed with T7 RNA polymerase to produce 118 nt poliovirus plus strand sequences. Alternatively, the plasmid was digested with ClaI and PstII, and extracted DNA was transcribed with SP6 RNA polymerase to produce 55 nt poliovirus minus strand sequences.

All transcription reactions (20 μl) contained 1 μl of 60 μM [3H]UTP to facilitate quantitation of transcripts made. Hybrid structures were then formed based on a 1 to 1 molar ratio of plus and minus strands for either the poliovirus 1–72 hybrid or for the poliovirus 5522–5627 hybrid in 100 mM NaCl to yield solutions of 40 pmol of hybrid per μl. Samples were heated at 65 °C for 30 min and then slow cooled to 37 °C over 25 min; these solutions could be stored at −20 °C. Hybrid formation was confirmed by restriction digests and gel analyses. "P" transcripts obtained from the transcribed hybrid in 6% polyacrylamide gels containing 7 μM urea and TBE buffer (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA, pH 8.3). Preparation of Purified Transcripts or Transcripts Synthesized from PCR-derived cDNA—T7 or SP6 transcripts, synthesized from DNA digested with the appropriate restriction enzymes, were fractionated in 6% polyacrylamide, 7 μM urea gels (TBE buffer) that were 1.5-mm thick at 8 watts (constant) until the bromphenol blue dye migrated about 80% to the bottom. The gel was then stained with ethidium bromide (0.5 μg/ml) in TBE buffer at room temperature for 30 min. Transcripts were visualized under ultraviolet radiation and appropriate transcripts were excised. These gel bands were dispersed through an 18 gauge needle, and RNA was extracted with 3 vol of 0.5 mM ammonium acetate, 10 mM EDTA, pH 7.5, at 4 °C overnight. The mixture was centrifuged, the supernatant collected, and RNA was precipitated with 2.5 vol of 100% ethanol; the concentration of the transcripts was determined by absorbance at 260 nm.

Defined transcripts were also obtained by transcription of PCR-generated cDNA. Using pGEM-4Z-PV1-72 for a template and the following primers, GEM-4Z-2488(+5522)+3 k5-GAGCCATGGTTACGACTAGTGGCCGAGCCGAGCGCTGG-3 and PV5522-5627(-5627)+3 AGCAGATTGTACTGAGAGTG-GGGA-3, cDNA was amplified and extracted, as described above. The cDNA was digested either with RsaI or with EcoRI, and the DNA was fractionated in a 1.5% agarose gel. The 108-fragment was isolated from the EcoRI-digested DNA, or the 285-fragment was excised from the RsaI-digested DNA. The DNA was extracted with the Qiaex II kit. The 108- and 285-fragments were transcribed with T7 RNA polymerase and SP6 RNA polymerase, respectively, with the Ambion MEGAscript kits, as above.

Preparation of Poliovirus 3D and 3AB—Poliovirus 3D polymerase was expressed in E. coli BL21(DE3) pLysS-pT7-3D cells (31) at room temperature in medium containing 16 g/liter tryptone, 10 g/liter yeast extract, 4 g/liter NaCl, 30 mM K2HPO4, and 5 mM glucose. pH 8.3, under ampicillin (100 μg/ml) and chloramphenicol (20 μg/ml) selection and were induced with 0.5 mM isopropyl β-D-thiogalactopyranoside and when the A600 reached 0.5, induction was continued for 20 h. RNA polymerase was purified from these cells essentially as described previously (6, 32), including preparation of a soluble lysate, 0–40% saturation ammonium sulfate precipitation, phosphocellulose chromatography, and two Mono Q column purifications.

Poliovirus 3AB was expressed in E. coli BL21(DE3)-pLysS-pT7lac3AB and purified as described by Lama and Carrasco (33) and Lama et al. (22). Both 3D and 3AB preparations were stored in Buffer A (50 mM Tris-HCl, pH 8.0, 50 mM KCl, 5 mM β-mercaptoethanol, 0.1% Nonidet P-40, and 10% glycerol) at −70 °C.

Assay of 3D Elongation Activity—Poliovirus 3D polymerase assay mixtures contained 50 mM HEPES, pH 8.0, 0.5 mM of each nonlabeled NTP, 10 μM of [32P]UTP or [32P]UTP (0.2 μCi/μl of assay mix), 4 mM dithiothreitol, 3 mM magnesium acetate, 0.8 μM 3H-hybrid or 3H-template, 1 unit/μl RNasin, plus or minus 0.72 μM 3AB, and variable amounts (3–600 nM, but usually 10 nM) of 3D. Reactions were started by the addition of 3D on ice, and aliquots (20 μl) were removed at zero time and after 30 min at 30 °C for acid precipitation. Precipitates were collected on Whatman GPC filters and washed with 1 M HCl, 0.1 M HCl followed by 1 M NaCl to yield solutions of 40 pmol of hybrid per μl. Samples were heated at 65 °C for 30 min and then slow cooled to 37 °C over 25 min; these solutions could be stored at −20 °C. Hybrid formation was confirmed by restriction digests and gel analyses.

The abbreviations used are: bp, base pair(s); PCR, polymerase chain reaction; nt, nucleotide(s).
sodium pyrophosphate and ethanol, dried, and counted in scintillation fluid.

Oxidation of Template Strands—3H-labeled T7 RNA polymerase-generated (−) strands (PV1-72) (1000 pmol) in a 50-μl reaction were incubated with 1 mM sodium periodate (Sigma) and 2 mM HCl at room temperature for 60 min in the dark. Then glycerol (0.68 M) was added for a further incubation at room temperature for 30 min in the dark to destroy excess periodate. Finally, the solution was diluted to 200 μl, containing 50 μg of yeast tRNA and 0.5 mM ammonium acetate, 10 mM EDTA, and RNA was precipitated with an equal volume of isopropanol, collected by centrifugation, washed with ethanol, and dried. The RNA was dissolved in 10 μl of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. This oxidized template was used directly in 3D reactions or annealed with [3H]SP6 (+) strands to make hybrid structures, as described above.

Analysis of Products of 3D Reactions—3D reactions with template alone or with hybrid substrates (PV1-72 or PV5522–5627) plus or minus 3AB were incubated, as described above. Aliquots (20 μl) were removed for analysis of native RNA products, supplemented with 50 μg of glycocholine (Boehringer Mannheim) or yeast tRNA carrier, and extracted with phenol-CHCl3. RNA was precipitated with isopropanol, washed with ethanol, and dried in a Speed-Vac (Savant). Pellets were dissolved in TBE buffer, 8 M urea, 0.025% bromphenol, 0.025% xylene cyanol prior to gel analysis.

RNA samples were glyoxalated to analyze RNA strands (34). Aliquots (20 μl) of 3D reactions supplemented with 50 μg of yeast tRNA were extracted, precipitated, and dried, as described above. Pellets were dissolved in a total of 30 μl containing 10 mM sodium phosphate, pH 7.0, 0.5% dimethyl sulfoxide (Fluka), and 1 mM glyoxal (Fluka) freshly deionized with Bio-Rad 501-x8(D), and incubated at 50 °C for 60 min. Samples were diluted with an equal volume of 0.8 M LiCl, and RNA was precipitated with 2.5 vol of ethanol. Precipitates were collected in a Biofuge 13 (Heraeus) at 4 °C, 13,000 rpm, 30 min, and dried; pellets were dissolved in TBE buffer, 8 M urea, 0.025% bromphenol, 0.025% xylene cyanol (TBE/urea sample buffer) prior to analysis in a polyacrylamide/urea gel, as described below.

RNase A digestion of aliquots (20 μl) from 3D reactions was used to determine lengths of duplex segments in RNA products. RNA samples were adjusted to 0.3 mM NaCl and digested directly with 20 μg/ml of RNase A (preheated in 0.1 mM sodium acetate, pH 5.2, at 80 °C for 10 min) at 30 °C for 30 min. Then glycojen carrier (50 μg) was added and samples were extracted with phenol-CHCl3, precipitated with isopropanol, dried, and finally dissolved in TBE/urea sample buffer, as described previously for gel analysis.

S1 nuclease digestion was performed by dilution of a 20-μl aliquot of a 3D reaction to 30 μl, containing 0.3 mM NaCl, 30 mM sodium acetate, pH 4.5, 3 mM ZnSO4, 10 μg/ml alkali-digested salmon sperm DNA, and 1 unit of S1 nuclease and incubating at 45 °C for 15 min. Samples were prepared for gel analysis, as described for RNase A-treated samples.

Proteinase K (Boehringer Mannheim) digestion of aliquots (20 μl) from 3D reactions was done by adjusting to 30-μl reactions containing 0.5% SDS, 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, and proteinase K (50 μg/ml) and incubating at 37 °C for 30 min. Thereafter samples were prepared for gel analysis, as described for RNase-treated samples.

Routine product analyses were in 6% polyacrylamide, 7 M urea gels in TBE buffer (20-cm wide × 14-cm long × 0.8-mm thick), and the bromphenol blue dye front was run about 80% down the gel at 12 watts (constant power). Under these conditions (samples were not boiled before application to the gel), stable RNA hybrids were not denatured in 7 M urea; only intrastrand secondary interactions were eliminated. After glyoxal treatment, RNA strands were fully separated and prevented from subsequent interaction. The gel was dried directly on Whatman 3MM sheets at 80 °C in a vacuum (Bio-Rad gel dryer). [32P]RNA bands were detected by autoradiography on Kodak BioMax MR film.

RESULTS

Characterization of a 3AB-stimulated 3D-catalyzed Reaction—A defined heteropolymeric substrate was constructed to facilitate interpretation of the analysis of products formed under various conditions and to allow systematic characterization of the reaction properties. The substrate, PV1-72, shown schematically in Fig. 1A, contained a partial duplex (25 bp) with two 5‘-overhangs of 11 and 63 nt. The shorter strand in the duplex included nt 1–21 of poliovirus plus strand RNA, and the longer strand included 72 nt of poliovirus minus strand RNA. The relative amounts of each strand required to form a hybrid were titrated; hybrid formation was monitored by polyacrylamide/urea gel electrophoresis. When used in 3D-catalyzed RNA polymerase reactions, the hybrid substrate was usually present at 0.8 μM, at which level the reaction rate was independent of substrate concentration.

Addition of 3AB to the reaction resulted in an approximately 10-fold stimulation of UMP incorporation (see below). In preliminary experiments, we confirmed that 3AB had no effect on chain elongation rates (24). The Km for NTPs or for hybrid substrate was unaffected by 3AB (Table I). In addition, the concentration of 3AB required to achieve maximal stimulation did not vary with the size of single-strand segments in the template over a 50-fold range (63 nt compared with 3000 nt, data not shown); thus 3AB did not appear to coat single-strand segments of the template to form a more active substrate for 3Dpol.

Fig. 2, lane 2, shows an autoradiogram of the products formed by poliovirus 3Dpol on the PV 1-72 substrate. The major product represents copying of the long 5‘-overhang to generate FIG. 1. Hybrid substrates used for poliovirus RNA polymerase reactions and the expected products. Schematic structures of RNA are depicted with bars for poliovirus sequences, lines for vector sequences, and wavy lines for 32P-labeled RNA strands filled in by the polymerase reaction. Connecting lines between strands denote hybridized segments. A, hybrid PV1-72 contains an 88-nt strand containing poliovirus minus strand sequences and a 96-nt strand containing plus strand sequences. Single-strand overhangs are 11 nt and 63 nt. Native products (1) and (2), denote fill-in with 32P-labeled segments on the unpaired overhangs. Detectable, radioactive strands (‘1) and (‘2) are derived from native products (1) and (2), respectively. B, hybrid PV5522–5627 contains a 118-nt strand containing poliovirus plus strand sequences and a 55-nt strand containing minus strand sequences. Single-strand overhangs are 72 nt and 9 nt. As in A, 32P-labeled native products, (1) and (2), and their labeled denatured strands, (‘1) and (‘2) are shown, respectively.
3AB Effects on Polio Polymerase Reaction

| Substrate | 3AB\(^a\) | \(K_m\) | \(V_{max}^b\) |
|-----------|-----------|--------|----------------|
| CTP       | —         | 4.0    | 1.8            |
| CTP       | +         | 2.6    | 1.6            |
| UTP       | —         | 1.0    | 1.2            |
| UTP       | +         | 2.4    | 0.8            |
| ATP       | —         | 5.0    | 0.5            |
| GTP       | —         | 2.5    | 1.2            |
| PV(1-72)  | —         | 0.09   | 0.4            |
| PV(1-72)  | +         | 0.24   | 1.3            |

\(^a\) 3AB at 0.72 \(\mu\)M, when present (+); when absent (-).

\(^b\) Assays were at 30 °C. Values are micromoles of NMP incorporated per nanogram of 3D.

![Table I](image)

**Fig. 2.** Products of poliovirus 3D\(^{pol}\) reactions in the absence and presence of 3AB. PV1-72 hybrid substrate, prepared from unpurified transcripts, was incubated with 15 nM 3D (lanes 1 and 2), 5 nM 3D and 720 nM 3AB (lanes 3 and 4), or 720 nM 3AB (lanes 5 and 6) for 0 min (lanes 1, 3, and 5) or 60 min (lanes 2, 4, and 6). Aliquots were removed at the indicated times, and RNA was purified and analyzed in a 6% polyacrylamide/7% urea gel. The gel was dried and autoradiographed.

**Fig. 3.** Native and denatured products from 3D reactions in the absence and presence of 3AB. Hybrid or template substrates were prepared from unpurified transcripts. After each 3D reaction of 30 min, samples (20 \(\mu\)l) were removed, phenol-CHCl\(_3\) extracted, and RNA was precipitated. \(^{32}\)P-Labeled products were untreated (lanes 1–4) or glyoxal-denatured (lanes 9–12) and fractionated in a polyacrylamide/urea gel and visualized by autoradiography. \(^{32}\)P-Labeled PV1-72 template (88 nt), primer (36 nt), and hybrid were analyzed in lanes 5, 6, and 7, respectively, as markers, and lane 8 contains denatured \(^{32}\)P-template.

A mostly double-stranded RNA product (Fig. 1A, (1) fill-in product, see below). In addition, a less abundant product of slightly lower mobility is produced. A fraction of the product (about 10%) remained in the sample well, representing either unaggregated or very large RNA product. This was not due to residual unincorporated nucleotides, since it formed during incubation (Fig. 2, lanes 1 and 2). Addition of 3AB to the reaction resulted in an 11-fold stimulation of activity (lane 4), although the vast majority of the stimulated product was large or aggregated material. (Note that less 3D\(^{pol}\) was utilized in the 3AB-stimulated reaction.) In addition, there was increased synthesis of the product with slightly lower mobility than the fill-in product. No product was formed by 3AB, in the absence of 3D\(^{pol}\) (lane 6).

Utilization of the template shown in Fig. 1A required a primer strand, as expected for 3D-catalyzed reactions, although a low level of primer-independent activity generated two bands plus some large or aggregated material (Fig. 3, lane 1). 3AB greatly stimulated production of these products plus additional ones (lane 3), suggesting an increased utilization of transient self-primed template structures. As seen above, in the presence of the hybrid substrate, 3D catalyzed the fill-in of unpaired single-strand segments to generate duplex products (Fig. 1A, (1) and (2)) in addition to some large, unresolved material (Fig. 3, lane 2). 3AB stimulated this reaction 5-fold, generating predominantly large products and lesser amounts of two duplex species (Fig. 1A, (1) and (2)), one of which was greatly enriched in the 3AB-stimulated reaction (Fig. 3, lane 4). If nonpoliovirus proteins (ovalbumin or cytochrome c) are substituted for 3AB in the 3D reactions, the products are the same as those formed with 3D alone, and there is no stimulation of activity or change in distribution of products (data shown below).

This initial characterization of products of the 3D reaction presents the following issues, which will be addressed below. (1) Stimulation of the 3D reaction by 3AB results in formation of large products. (2) In reactions containing 3D plus 3AB, a second product migrating slower than fill-in product is preferentially enhanced. (3) The addition of 3AB to a 3D-catalyzed reaction greatly amplifies a primer-independent reaction.

Characterization of Large Products Formed in the Presence of 3AB—Denaturation of the products from a reaction containing 3AB (Fig. 3, lanes 11 and 12) with template alone or hybrid substrates had little or no effect on the mobility of the predominant large products. As previously noted under “Experimental Procedures,” glyoxal treatment was essential to fully denature RNA-RNA duplexes and prevent subsequent strand interactions. The persisting large products were, therefore, not the result of aggregation of unit length strands. In reactions with template alone, the same denatured strands were observed in the 3D reaction with and without 3AB, although 3AB stimulated the reaction considerably (lanes 9 and 11); thus 3AB appears to facilitate utilization of terminal 3’-hydroxyls as primers for elongation, perhaps stabilizing short snapback structures.

To examine the possibility that large product was formed by
Hybrid and template substrates were prepared from unpurified PV1-72 transcripts. Oxidized templates were used in the substrates in lanes 4–6. 32P-Labeled products from 3D-catalyzed reactions with or without 3AB were obtained after 30 min of incubation; RNA was extracted, precipitated, and fractionated in a polyacrylamide/urea gel and was detected by autoradiography. Lane 7 contains marker 32P-labeled unoxidized template (88 nt), and lane 8 contains marker 32P-labeled hybrid (unoxidized template strand).

end addition to the template strand, we prepared 32P-hybrid substrate for a 3D-catalyzed reaction with unlabeled nucleotides in an effort to observe conversion of starting template to large product. Very little substrate is converted to product, however, even in the presence of high concentrations of 3D, so no significant accumulation of labeled product was detected (data not shown). As an alternate approach, we oxidized the 3′-hydroxyl of the template strand with periodate to prevent end addition and examined large product formation (Fig. 4). In primer-independent reactions with 3D plus 3AB (Fig. 4, lanes 3 and 6), large products were not synthesized on the oxidized template (lane 3). However, with a hybrid substrate, large products were formed with or without an oxidized template strand (lanes 2 and 5). In this case, synthesis must have occurred by addition to primer strands. The end addition reaction is dependent on 3AB since with 3D alone using the hybrid substrate, the expected fill-in products (Fig. 1A, (1) and (2)) were synthesized with or without oxidized template strands, and there was little accumulation of large products (lanes 1 and 4). These data suggested that end addition to RNA present in either the template or primer preparations generated the very large product RNAs. Examination of 32P-labeled template, primer, and hybrid extracted directly from the transcription mix revealed the presence of a small population of contaminating long RNAs (Fig. 3, lanes 5–8 and Fig. 4, lanes 7 and 8). These transcripts are likely generated from a small amount of DNA that remains uncut after intended linearization. Interestingly, this subpopulation of RNA is efficiently recognized by 3AB and thereby apparently made available for elongation by 3D. Their length and possible snapback or other structural folds may provide multiple opportunities for use as templates, especially with 3AB interaction and allows for large amounts of labeled nucleotide incorporation into small molar quantities of product.

Reactions Utilizing Hybrids Derived from Purified Transcripts—To test whether contaminating long RNAs were the source of the large products, unit-length transcripts (88 nt and 36 nt) were purified from preparative acrylamide gels and used to form hybrid substrates for 3D. The PV1-72 hybrid was used in reactions with 3D alone (Fig. 5, lanes 1–4) or with 3D plus 3AB (lanes 5–8). With these purified RNAs, there was no stimulation of 3D activity by 3AB, and large products were not formed in the presence or absence of 3AB. These results confirmed the prediction that the large products were formed by end addition to contaminating transcripts. The reaction with 3D plus 3AB still yielded two different species of product (Fig. 5, lanes 5–8; Fig. 1A, (1) and (2)). A second method to prepare transcripts without contaminating long RNA strands was investigated. PCR-generated cDNA, which contained the coding sequences for selected RNAs (108 bp and 285 bp for minus and plus strand transcripts, respectively), was prepared for transcription reactions to yield RNA for the formation of the same PV1-72 hybrid structure (Fig. 6, lanes 1 and 2). Under similar reaction conditions where the unpurified transcripts of linearized plasmids yielded large products in reactions with 3D plus 3AB (Fig. 6, lanes 3 and 4), transcripts obtained from PCR-derived cDNA gave hybrid substrates that did not yield large products with 3D plus 3AB and demonstrated no 3AB stimulation of 3D activity (lanes 5–7). Thus, large products are due to contaminating RNAs that form potential templates in primer-independent reactions; stimulation by 3AB is due to enhanced recognition and utilization of these potential substrates.

3AB Stimulation of the 3D Reaction as a Function of 3D Concentration—Other investigators have reported maximal 3AB stimulation of 3D activity at low 3D concentrations using a heteropolymeric hybrid or template substrate (22–24). We examined 3AB stimulation as a function of 3D concentration.
between 3 nM and 600 nM 3D. 3AB added to hybrid derived from unpurified transcripts of linearized plasmids gave increasing stimulation with decreasing 3D concentration with a maximum of 5–10-fold stimulation at 3 nM 3D in repeated experiments (Fig. 7). Above 30 nM 3D there was no 3AB stimulation. With hybrid substrate derived from purified transcripts there was no 3AB stimulation over the entire 3D concentration range, supporting the conclusion that 3AB stimulation occurred on containing primer-independent templates. Furthermore, 3AB does not serve to stabilize active 3D at low protein concentrations.

Characterization of the Slower Mobility Species Induced by 3AB—A second characteristic of the 3AB-stimulated reaction is the marked enrichment of a product migrating as a discrete band slightly slower than the fill-in product (Fig. 1A, (1); e.g. Fig. 3, lane 4; Fig. 5, lanes 5–8; Fig. 6, lanes 4 and 11). To characterize this slower mobility species, the products of a 3D plus 3AB reaction with PV1-72 hybrid substrate, formed from transcripts synthesized from PCR-generated cDNA, were subjected to various treatments (Fig. 8). The previously observed fill-in products were formed in a reaction with 3D alone (Fig. 8, lane 1; Fig. 1A, (1)); the predominant product co-migrates, coincidentally, with the template strand (lane 5). The same fill-in products, plus a slower migrating species, were formed in the reaction with 3D plus 3AB (Fig. 8, lane 2). (The formation of minor amounts of larger RNA species, generated by end addition to unhybridized template or primer strands that form transient snapback structures apparently stabilized by 3AB, can also be seen in this gel.) When these products were denatured with glyoxal, unit-length strands were formed for each product (Fig. 8, lanes 3 and 4; Fig. 1A, (1’) and (2’)). Thus the slower migrating species formed with 3D plus 3AB had only unit-length 32P-labeled strands. S1 nuclease digestion of products was incomplete (lanes 8 and 9) but generated for both reactions RNA with a faster mobility. Single-strand segments were completely digested with RNase A in high salt and showed, almost exclusively, a single duplex segment for RNA derived from the 3D reaction (lane 10), consistent with the presence of the longer 5’-overhang of the hybrid substrate (Fig. 1A, (1)). In contrast, the products from the 3D plus 3AB reaction contained two major species of duplex segments, consistent with the fill-in at either 5’-overhang in the hybrid but only one overhang per molecule to yield duplexes of 88 and 36 bp (lane 11; Fig. 1A, (1) and (2), respectively). This result further supports the in-
3AB Effects on Polio Polymerase Reaction

**Fig. 9.** Effect of various proteins on 3D-catalyzed reactions with hybrid or template alone. Transcripts for substrates were synthesized from PCR-amplified cDNA. After a 3D reaction of 30 min with the indicated supplements, samples (20 μl) were removed, phenol-CHCl₃ extracted, and RNA was precipitated. The resulting ³²P-labeled products were analyzed in a polyacrylamide/urea gel by autoradiography. Samples in lanes 1–4 were from reactions containing template substrate alone. ³²P-Labeled marker RNAs are template (lane 5, 88 nt), primer (lane 6, 36 nt), hybrid (lane 7), and two times unit length template (lane 8, 176 nt).

Interpretation that 3AB increases utilization of 3'-hydroxyls, which appear unavailable for utilization as primer for 3D alone. Finally, proteinase K digestion of products did not alter the mobility of products (compare Fig. 8, lanes 1 and 2 with lanes 12 and 13). Therefore 3AB association with RNA products does not survive phenol-CHCl₃ extraction and does not account for the slower mobility species associated with the 3D plus 3AB reaction.

These results show that the slower mobility species enriched among the native products of the 3D plus 3AB reaction contains an extension of the minus strand of the PV1-72 hybrid with the starting plus strand unaltered (Fig. 1A, (2)). After denaturation only minus strand from this species contains ³²P and hence denaturation yields full-length strands (Fig. 1A, (2')).

**Primer-independent Reaction in the Presence of 3D Plus 3AB**—As shown above, hybrid substrate prepared from transcripts of PCR-generated cDNA produced no large products and no stimulation of 3D activity by 3AB, although there was increased utilization of the minus strand 3'-hydroxyl to produce the partially duplex product with reduced mobility (Fig. 9, lanes 1 and 2). Previous data (Fig. 3, lanes 1 and 3) also showed that addition of 3AB to a 3D-catalyzed reaction greatly stimulated utilization of template as substrate in the absence of primer. Fig. 9 (lanes 9 and 10) illustrates this stimulation of primer-independent 3D activity, using PCR-generated cDNA transcripts to prevent generation of large RNA products. With a substrate of template alone, the same products were made in a 3D reaction (Fig. 9, lane 9) as in a 3D reaction supplemented with 3AB (lane 10), ovalbumin (lane 11), or cytochrome c (lane 12), but only 3AB specifically stimulated synthesis of each of the products, confirming that 3AB promotes utilization of 3'-hydroxyls that may form transient intramolecular base pairs. 3AB may stabilize these structures by binding RNA, as well as binding 3D to create a potential site for elongation.

3AB Effects on the 3D Reaction Using a Different Hybrid Substrate—Another hybrid substrate, PV5522–5627 (Fig. 1B), was prepared to ensure that the 3AB effects on the 3D reaction were not unique to the PV1-72 substrate. This substrate also contained 5'-overhangs that potentially could be filled in with 3D in elongation reactions. The poliovirus RNA sequences were derived from a portion of the 3C coding region. Using gel-purified transcripts for preparation of hybrid and template substrates, there was no accumulation of large products and no 3AB stimulation of 3D elongation activity (Fig. 10, lanes 1–4 and 9–12). Reaction with hybrid substrate gave fill-in product (lanes 2 and 4; Fig. 1B, (1) and (2)), which upon denaturation gave predominantly unit-length strands with or without 3AB, as before (lanes 10 and 12; Fig. 1B, (1') and (2')). The strands were 9 nt longer than the starting template strand because the primer strand has a 9-nt extension beyond the 3'-end of the template. In an identical experiment using crude transcripts for preparation of hybrid and template substrates, large products comprised the predominant product and 3AB stimulated the 3D reaction 12-fold with a hybrid substrate (data not shown). Thus, the same pattern of results was observed for both substrates.

However, some differences were observed with the two substrates. For example, PV5522–5627 template alone produced very little product with or without 3AB (Fig. 10, lanes 1 and 3 or lanes 9 and 11), suggesting that no transient snapbacks are formed that could be stabilized by 3AB. In addition, little or no second product with slightly slower mobility was formed or enriched by 3AB supplementation of the 3D reaction with the PV5522–5627 hybrid substrate (Fig. 10, lanes 2 and 4). In some experiments, some slower mobility product was detected (Fig. 11, lane 2). Thus, 3AB appeared to only poorly promote utilization of the 3'-hydroxyl of the plus strand in this hybrid over and above what 3D could do alone.

Additional characterization of the products of the PV5522–
Evidence for the role of 3AB in poliovirus RNA replication stems from observation of the RNA-negative phenotype conferred by mutations in either the 3A or 3B coding regions, from biochemical observations of 3AB associated with intracellular replication complexes, and from the presence of 3B on incomplete nascent RNA strands. These data prompted several recent efforts to examine the effects of 3AB on various putative steps in the RNA replication reaction. For example, it was shown that 3AB interacts with RNA (26, 27) and with 3CD and 3D (24, 25, 36) and that it can form a complex with 3CD and the termini of poliovirus plus strand RNA (26, 35). In addition, several investigators have reported that 3AB stimulates 3D polymerase activity (22–24, 27). The biological relevance of these observations to the viral RNA replication mechanism has yet to be determined.

In this report, we have examined the stimulation of 3D-catalyzed RNA synthesis by 3AB in some detail. We conclude that 3AB does not stimulate 3D activity on a template that is stably base paired to a primer. Thus, 3AB has no inherent effect on 3D stability or catalysis. If no primer is available or if the primer is not stably base paired to the template, 3AB stimulates 3D activity significantly. We postulate that 3AB interacts with 3′-hydroxyl ends that are transiently base paired, resulting in stabilization of a hybrid region containing a site for elongation. Recruitment of 3D to the site by protein-protein interaction would result in increased elongation activity. As predicted, stimulation is greatest when 3D concentration is low, since 3AB-3D interactions would have a greater impact on localizing 3D to the initiation site than when 3D is in excess. The hypothesis that 3AB-3D interaction is responsible for the increased polymerase activity is consistent with our recent observations that 3AB can stimulate polymerase activity catalyzed by the closely related Coxsackievirus B3 RNA polymerase but has no effect on polymerase activity catalyzed by avian myeloblastosis virus reverse transcriptase on the same template (data not shown).

Templates with high degrees of internal structure frequently fail to form stable hybrids with primer sequences even after denaturation and renaturation in primer excess. We have examined “hybrids” intended for use as substrates in the 3D reactions in polyacrylamide/urea gels. Although the PV1-72 substrate, used extensively in our studies, produced very stable hybrids, we were unable to generate a stable hybrid using the entire 5′-terminal cloverleaf structure (37) spanning nt 1–108. Similarly, stable hybrids did not form from the 3′-end of the poliovirus genome with transcripts from nt 7332 through the poly(A) segment into vector sequences and a variety of primers spanning the poly(A) tail with heteronucleotides on either side. It is likely that heteropolymeric substrates used by others (23, 24), which were reported to support significant stimulation of 3D activity by 3AB, were unstable hybrids, behaving in a fashion similar to the primer-independent reactions described here. In addition, poly(A)-oligo(dT) substrate produced a 50–100-fold stimulation of the reaction by 3AB (22, 23). This large stimulation may result from transient hybrid formation on a template with multiple potential start sites.

The large structures formed in 3D reactions supplemented with 3AB have been observed previously (23). We have shown that these products are synthesized from contaminating transcripts produced by SP6 and T7 RNA polymerases from a population of DNAs. To form functional hybrid substrates for 3D reactions without production of large products, transcripts must be gel-purified or cDNAs must be constructed of small size and these must be gel-purified prior to transcription with SP6 or T7 RNA polymerase. We have repeatedly found that DNA digested with restriction endonucleases, seemingly to
completion by analytical gel analysis, does not produce a homogenous population of transcripts. The large products are favored in 3D plus 3AB reactions since they are primer-independent and because radioactive incorporation into product is length-dependent. 3D alone does not amplify production of large products because transient hybrid structures that include the 3'-hydroxyl terminus are apparently not accessible to 3D at the concentration used in the absence of 3AB.

The primary product synthesized by 3D alone on the PV1-72 substrate was a fill-in complementary to the longer 5'-overhang (Fig. 1A, (1)). Addition of 3AB enriched for a slower mobility band, which represented fill-in complementary to the shorter 5'-overhang (Fig. 8, lanes 10 and 11; Fig. 1A, (2)). There is some indication (Figs. 3 and 5) that both termini are not utilized simultaneously, perhaps merely due to the low statistical probability of one molecule being bound twice in a reaction where overall template utilization is extremely low.

The biochemical roles for 3AB in the process of viral RNA replication in infected cells are yet undefined. Certainly its potential to serve as the VPg donor for RNA strand initiation is critical. Its association with intracellular membranes (16–18) may anchor the replicating structures in the replication complex. Its ability to bind RNA, whether or not the interactions are sequence-specific, may facilitate positioning of 3AB on the replicating RNAs. The activity described in this report may result in stabilization of the weak hybrid formed between the template and a uridylylated form of 3AB or 3B. Finally, affinity for 3CD and stimulation of cleavage to generate 3D or direct template and a uridylylated form of 3AB or 3B. Finally, affinity for 3CD and stimulation of cleavage to generate 3D or direct

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