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Host Factor-Induced Template Modification during Synthesis of Poliovirus RNA In Vitro

TIMOTHY D. HEY,[1]† O. C. RICHARDS,[1,2] AND E. EHRENFELD[1,2]*

Department of Cellular, Viral and Molecular Biology[1] and Department of Biochemistry,[2] University of Utah School of Medicine, Salt Lake City, Utah 84132

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Poliovirus RNA polymerase, $3D^{pol}$, transcribes poliovirus RNA in vitro in the presence of a host factor (HF) preparation from uninfected HeLa cells to yield heterogeneous-size product RNAs. The products include some molecules larger than the template that represent self-primed elongations of the template from a 3′-terminal hairpin. We showed that transcription proceeded through the formation of a modified RNA intermediate that was generated by nucleolytic cleavage of the template by HF in the absence of nucleoside triphosphates. Cleavage resulted in the loss of the original poly(A) 3′ end and the generation of new, heterogeneous 3′ ends that formed self-priming structures that could then be elongated by $3D^{pol}$ or reverse transcriptase. The two stages of the reaction, (i) cleavage to yield self-priming templates and (ii) subsequent chain elongation to yield heterogeneous-size products up to nearly dimer length, could be separated. RNAs whose original 3′ ends were chemically oxidized so as to prevent chain elongation showed no reduction in template activity after preincubation with HF. We conclude that an HF preparation that contains a low level of nuclease activity is sufficient to activate RNA templates for transcription by $3D^{pol}$ to generate up to apparent dimer-length products. This reaction likely has little relevance to the mechanism of poliovirus RNA replication in vivo. It is likely that numerous other factors or activities, in addition to $3D^{pol}$, would also result in transcription of poliovirus RNA in vitro.

Poliovirus is a single-stranded RNA virus that is a member of the picornavirus group. Its 7,433-nucleotide genome is of positive polarity, and the complete nucleotide sequence has been determined for members of each of the three virus serotypes (21, 30, 32, 34). The virion RNA has a heterogeneous poly(A) tract (38) at its 3′ end and a small viral protein, VPg (18, 22), covalently linked to its 5′ end.

Replication of the poliovirus genome occurs in a membrane-associated complex in the cytoplasm of infected cells (9). Studies to elucidate the mechanism of this RNA-dependent RNA synthesis have followed two different paths. One approach has been an analysis of several activities that can be detected in a crude, membrane-associated complex, isolated from virus-infected HeLa cells, that contains endogenous RNA template and synthesizes full-length plus-strand product RNAs in vitro. Recent work has shown that VPgpU and VPgpUpU are also synthesized in vitro by the crude replication complex and that at least some of these molecules can be extended to VPgpUUAAAACAG (33). It is hoped that further dissection of the various activities in this replication complex will identify the protein components required for poliovirus RNA replication.

The second approach has been based on attempts to reconstitute a replication system in vitro from purified protein and RNA components. This approach was dependent upon the purification of the viral polymerase, $3D^{pol}$ (nomenclature of Rueckert and Wimmer [31]), in a template-dependent form (13, 16, 17). When this was accomplished, it became apparent that $3D^{pol}$ catalyzes only RNA chain elongation; for example, poly(A)$^+$ poliovirus RNA template is transcribed by this enzyme only if a synthetic oligo(U) primer is provided (16, 17, 19, 35, 36). Subsequent work was directed at identifying initiation factors for RNA synthesis which, it was postulated, would act in concert with $3D^{pol}$ to initiate, as well as elongate, RNA chains. The strategy used was to assay fractions of cell extracts for their ability to stimulate RNA synthesis by $3D^{pol}$ in the absence of an oligonucleotide primer. Such activity was found (15); it was present in uninfected HeLa cells and thus was designated host factor (HF). Purification of HF proceeded in several laboratories, always based on its ability to permit $3D^{pol}$ to transcribe poliovirus RNA template. However, when the products generated by HF-mediated reactions in different laboratories with different preparations of $3D^{pol}$ were examined, quite different results were reported. Dasgupta (12) found RNA products that were complementary to the plus-strand poliovirus RNA template and were heterogeneous in length, up to the full size of the template. Some of these molecules precipitated with anti-VPg antibodies and appeared to be directly linked to VPg or related proteins (26, 28, 29). Similar results were reported by Baron and Baltimore (6, 8). The HF preparation used by Morrow et al. (27) was a highly purified 67-kilodalton protein that copurified with a protein kinase activity. In contrast, Young et al. (40) reported product RNA from their HF-mediated reactions that was up to twice the length of the template RNA. The larger product RNA resulted from addition of nucleotides directly onto the template to produce a full-length hairpin with complementary RNA sequences. No VPg sequences were directly associated with product RNA, and if VPg was removed from the template before transcription, no product could be immunoprecipitated with anti-VPg antibody (3, 39). A plausible mechanism for the generation of these dimer-size molecules was proposed by Andrews et al. (2, 4). They described the purification of a 68-kilodalton terminal uridylyltransferase (TUT) that also has HF activity. No data regarding the size or properties of product RNAs transcribed from poliovirus RNA template by $3D^{pol}$ and TUT have been

* Corresponding author.
† Present address: Section of Genetics, Cornell University, Ithaca, NY 14853.

reported. However, it has been proposed that the dimer-size molecules described by Young et al. (40) could occur by the terminal addition of uridylate residues to the 3′ poly(A) tract of the template; formation of a 3′-terminal hairpin, perhaps stabilized by HF, would then provide a self-priming sequence for chain elongation by $3D^{pol}$ (4).

In our laboratory, a relatively crude HF preparation has been used in conjunction with $3D^{pol}$ to generate product RNAs. We have reported products larger than the template and apparently identical to those reported by Young et al. (40). In addition, we have shown that HF-mediated product RNA is of both plus- and minus-strand polarity, shown by hybridization to single-stranded poliovirus DNAs (20).

The relevance of these various HF preparations and the products they generate in vitro to the mechanism of poliovirus RNA replication in vivo is yet to be demonstrated. To understand the activity of the HF preparation used in our laboratory, we analyzed the 3′ ends of the RNA templates that are used for chain elongation by $3D^{pol}$. This study demonstrated that random nucleolytic cleavage of the template by our HF preparation served to activate it for elongation by $3D^{pol}$. Activation presumably represented cleavage of the RNA chain to produce a terminal hairpin structure that could self-prime. Template elongation then occurred onto heterogeneous 3′ ends and did not occur from the normal 3′ poly(A) end of intact templates. The formation of product RNAs that were apparently twice the length of the template occurred by elongation of templates that had been cleaved quite close to the 3′ end. We demonstrated that this HF activity was sufficient to mediate the synthesis of products that were longer than templates by the viral RNA polymerase, and we conclude that the HF-induced RNA transcription occurring in vitro in this system is not likely to be involved in poliovirus RNA replication in vivo.

MATERIALS AND METHODS

Materials. Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co., St. Louis, Mo. Agarose was from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Oligo(U) was from Collaborative Research, Inc., Waltham, Mass. Sephadex G-50, S1 nuclease, T4 RNA ligase, and oligo(dT) cellulose were from Pharmacia Molecular Biologicals, Piscataway, N.J. Reverse transcriptase was from Life Sciences, Inc., St. Petersburg, Fla. Methyl mercury hydroxide was from Alpha Products, Danvers, Mass. [^{32}P]pCp was from Amersham Corp., Arlington Heights, Ill. All other radionuclides were from New England Nuclear Corp., Boston, Mass.

Cells and virus. Growth of HeLa S3 cells and purification of the Mahoney strain of poliovirus type 1 have been described (10).

Purification of poliovirus polymerase and HF. Purification of $3D^{pol}$ from poliovirus-infected HeLa cells has been described (20) and results in a preparation approximately 80% pure. Purification of HF from uninfected HeLa cells has also been described (20).

Polymerase assay conditions. The polymerase was assayed under three conditions that varied only in the added template and primer. Standard reaction conditions were as follows. A total of 5 μl of polymerase (approximately 50 ng of protein) was assayed in a 50-μl reaction mixture containing 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 8.0), 500 μM each ATP, CTP, and GTP, 4 mM dithiothreitol (DTT), 3 mM $Mg(CH_3COO)_2$, and 60 μM $ZnCl_2$. [α-^{32}P]UTP was the labeling nucleotide and was present at 11.4 μM with a specific activity of 10^5 dpm/pmol. Oligo(U)-dependent RNA transcription was monitored with 1 μg of poliovirus RNA in the presence of 0.034 μg of oligo(U). HF-dependent RNA transcription was measured with 1 μg of poliovirus RNA as the template and 5 μl of HF (approximately 5 μg of protein). HF-pretreated RNA transcription activity was tested with 1 μg of preincubated RNA in the absence of oligo(U) or HF. All assays were done at 30°C for 60 min.

HF-pretreatment reaction. Poliovirus RNA (1 to 5 μg) was incubated with 5 to 15 μg of HF (no polymerase) in a 50-μl reaction mixture containing 50 mM HEPES (pH 8.0), 500 μM each ATP, CTP, and GTP, 11.4 μM UTP, 4 mM DTT, 3 mM $Mg(CH_3COO)_2$, and 60 μM $ZnCl_2$. The mixture was allowed to incubate for 30 to 60 min at 30°C; the RNA was then extracted with phenol and precipitated with ethanol. Mock HF pretreatment was performed under identical conditions but without added HF.

Reverse transcription of poliovirus RNA. Mock-HF-pretreated poliovirus RNA or HF-pretreated RNA (1 μg) was incubated in a 50-μl reaction mixture containing 10 mM Tris hydrochloride (pH 8.3), 140 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, and 1 mM each dCTP, dGTP, and dTTP; [α-^{32}P]dATP (10^4 dpm/pmol) was the labeling nucleotide and was present at 10 μM. Oligo(U) primer was present at 1.4 μg/ml in some reaction mixtures. Reverse transcriptase (20 U) was assayed at 37°C for 60 min per reaction.

Denaturing agarose gel electrophoresis. Denaturing agarose gel electrophoresis of RNA has been described (5, 20).

End labeling and nearest-neighbor analysis. End labeling with $3D^{pol}$ was performed under standard reaction conditions except that GTP, CTP, and ATP were excluded from the reaction mixture. [α-^{32}P]UTP was present at 2 μM with a specific activity of 1.5×10^6 dpm/pmol.

End labeling with [^{32}P]pCp and RNA ligase was done in a total volume of 40 μl containing 0.3 to 1 μg of RNA. The reaction mixture consisted of 50 mM HEPES (pH 8.0), 3 mM DTT, 8 mM $MgCl_2$, 10% dimethyl sulfoxide, and 20 μM ATP. [^{32}P]pCp was present at 0.8 μM (3,000 Ci/mmol), and 4 U of T4 RNA ligase was used for each reaction. Samples were incubated on ice for 16 to 24 h. The reaction routinely labeled approximately 99% of the input RNA molecules.

End-labeling reactions were terminated by extraction with phenol. The aqueous phase was chromatographed through two Sephadex G-50 spin columns, as described previously (24), in 0.2 M LiCl–0.03 M Tris hydrochloride (pH 7.4)–2 mM EDTA–0.1% sodium dodecyl sulfate; the RNA in the excluded volume was then precipitated in ethanol, and the RNA pellet was dissolved in sterile distilled water. Gel filtration removed all detectable unincorporated nucleotides as assayed by high-voltage paper electrophoresis. Samples (1.0×10^4 to 5.0×10^4 cpm) were adjusted to a total volume of 20 μl containing 5 μg of yeast RNA carrier and 10% piperidine. The samples were sealed in capillary tubes and incubated at 90°C for 2 h. After hydrolysis, the samples were spotted onto 3MM filter paper (Whatman, Inc., Clifton, N.J.) and dried. The paper was brushed with a solution of 5% acetic acid–0.5% pyridine–1 mM EDTA (pH 3.5) and placed immediately into a Savant LT20A apparatus (Savant Instruments, Inc., Hicksville, N.Y.) containing the buffer described above. High-voltage paper electrophoresis was performed at 2,000 V until a xylene cyanol marker migrated 8 cm. The chromatogram was soaked in 95% ethanol–1% NH_4OH, dried, and exposed to XAR film (Eastman Kodak Co., Rochester, N.Y.) at −70°C by using a Cronex Light-

ning-Plus (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) intensifying screen.

Fractionation of HF-pretreated RNA on oligo(dT) cellulose. Approximately 1 g of oligo(dT) cellulose was hydrated in ET (10 mM Tris hydrochloride [pH 7.4], 1 mM EDTA) and poured into a column (0.7 by 20 cm). The resin was washed successively at room temperature with 5 ml each of ETF (10 mM Tris hydrochloride [pH 7.4], 1 mM EDTA, 50% formamide), ET, 0.1 M NaOH, ET, and finally ETN (10 mM Tris hydrochloride [pH 7.4], 1 mM EDTA, 1 M NaCl). The column was stored in ETN until ready for use. The HF-pretreated RNA (60 μg in 100 μl of water) was adjusted to 15 mM methyl mercury hydroxide and incubated at room temperature for 10 min. The RNA was diluted to 3 ml with ETN and loaded onto the column. The flowthrough [poly(A)$^-$] was collected, and the column was washed with 6 ml of ETN. Poly(A)$^+$ RNA was eluted with 3 ml of ET. The flowthrough, wash, and eluted fractions were precipitated in 70% ethanol overnight at −20°C. The precipitates were collected by ultracentrifugation in an SW41 rotor (Beckman Instruments, Inc., Fullerton, Calif.) at 30,000 rpm for 1 h at 4°C. The precipitates were suspended in water and analyzed by denaturing agarose gel electrophoresis for RNA content. The flowthrough and eluted fractions contained approximately 90% of the RNA, with the wash accounting for the remaining 10%.

Preparative methyl mercury hydroxide agarose gel electrophoresis. HF-pretreated or mock-HF-pretreated poliovirus RNA (10 to 30 μg) was loaded onto a 1% low-melting agarose gel containing 10 mM methyl mercury hydroxide. The electrophoresis conditions have been described (20). After electrophoresis, marker lanes were cut from the gel and stained in 0.5 M ammonium acetate containing 1 μg of ethidium bromide per ml. With markers as a guide, full-length poliovirus RNA was cut from the gel and melted in 3 ml of TNE (0.1 M NaCl, 0.01 M Tris hydrochloride [pH 8.4], 0.005 M EDTA) at 75°C. When melting was complete, an equal volume of TNE-saturated phenol was added, also at 75°C. The aqueous phase was extracted twice with phenol and then once with chloroform. The aqueous phase was adjusted to 0.2 M sodium acetate, and the RNA was precipitated with 2.5 volumes of 95% ethanol.

Oxidation of poliovirus RNA. The method of Marsh and Pace (25) was used to oxidize the 3′ end of poliovirus RNA. Briefly, 20 μg of poliovirus RNA was mixed with a 100-fold molar excess of sodium metaperiodate in a total volume of 20 μl. The periodate was added in the dark, and the sample was incubated at room temperature for 1 h in the dark. The sample was then diluted to 200 μl with H_2O, adjusted to 0.3 M sodium acetate (pH 5.0), and precipitated with 3 volumes of 95% ethanol.

RESULTS

Separation of 3D^{pol} and HF reactions. The products of transcription of poliovirus RNA by 3D^{pol} with an oligo(U) primer or HF have been described and are shown in Fig. 1, lanes 1 and 2. Without an oligo(U) primer or HF, 3D^{pol} is not capable of transcribing the template (Table 1 and Fig. 1, lane 3). Oligo(U) priming markedly stimulates incorporation of ribonucleotides (Table 1) and production of complementary product RNA up to template length (Fig. 1, lane 1) (8, 12, 20, 37). HF-mediated transcription gives some products larger than the template, apparently initiated from a template hairpin self-priming mechanism that has been described (Fig. 1, lane 2) (20, 40).

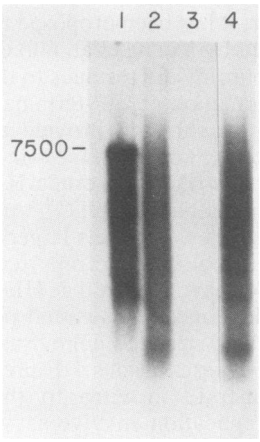

FIG. 1. Analysis of labeled product RNA by CH_3HgOH-agarose gel electrophoresis. Product RNA labeled with [^{32}P]UMP was synthesized under standard reaction conditions, extracted with phenol, and precipitated with ethanol before analysis by denaturing agarose gel electrophoresis. Poliovirus RNA was used as the template in the presence of oligo(U) (lane 1), HF (lane 2), or neither (lane 3). HF-pretreated template transcribed in the absence of oligo(U) or HF yielded the labeled RNA in lane 4. The position of full-length poliovirus RNA (7,500 nucleotides) was determined by ethidium bromide staining of virion RNA markers in adjacent lanes.

The activity of HF during transcription of poliovirus RNA by 3D^{pol} in vitro and its role in generating products that are elongated from the template are unknown. In some reactions, evidence has been presented to suggest that HF interacts directly with 3D^{pol} (7, 14); other models require that HF acts upon the RNA, making it a suitable template for 3D^{pol} (4, 40). In an effort to identify the site of action of HF, we attempted to separate the HF reaction from the RNA polymerization reaction by pretreating poliovirus RNA with HF in the absence of 3D^{pol}. HF was removed and RNA was purified by phenol extraction and ethanol precipitation, and the RNA was tested for its activity as a template for 3D^{pol} in the absence of any primer. The HF-pretreated RNA was actively transcribed by 3D^{pol} without an oligo(U) primer or any further addition of HF (Table 1). Mock-HF-pretreated RNA (without HF) was, as expected, inactive (Table 1). Product RNA from the HF-pretreated template resembled product RNA from HF-mediated transcription when examined by denaturing agarose gel electrophoresis (Fig. 1, lane 4). HF-pretreated template RNA maintained its primer-

TABLE 1. Activities of poliovirus RNA templates in poliovirus RNA polymerase reaction[a]

Template	Primer	Amt of [^{32}P]UMP incorporated (pmol/50-μl reaction)	Stimulation (fold)
Poliovirus RNA		0.08	
	Oligo(U)	5.2	65
	HF	0.77	10
HF-pretreated poliovirus RNA		1.2	15
Mock-HF-pretreated poliovirus RNA		0.05	

[a] The reaction conditions were described in Materials and Methods. Labeled product RNA was precipitated with 7% trichloracetic acid, collected on glass fiber filters, and analyzed for radioactivity.

TABLE 2. Activity of HF-pretreated poliovirus RNA templates with reverse transcriptase[a]

Poliovirus RNA template	Primer	Amt of [^{32}P]dAMP incorporated (pmol/50-μl reaction)	Stimulation (fold)
Mock HF pretreated		1.8	
	Oligo(U)	59	33
HF pretreated		19	11

[a] The reaction conditions were described in Materials and Methods, and labeled RNA was analyzed as described in Table 1, footnote *a*.

independent activity after successive phenol extractions, treatment with 1% sodium dodecyl sulfate, and digestion with pronase (data not shown). These results indicate that the ability of the HF-pretreated RNA to be transcribed by 3D^{pol} in the absence of oligo(U) primer or added HF was due to an RNA modification and not protein contamination from the HF-pretreatment reaction. The HF-pretreated RNA was also actively transcribed by reverse transcriptase in the absence of added primers (Table 2).

Requirements for activation of poliovirus RNA by HF pretreatment. The minimum requirements for HF pretreatment of poliovirus RNA were determined and are shown in Table 3. Mg^{2+} and HF were the only strict requirements of the reaction. Surprisingly, template activation [i.e., the ability to be transcribed in the absence of oligo(U) or HF] occurred in the absence of nucleoside triphosphates. This is inconsistent with the suggestion that a TUT is responsible for the HF activity described here, although the possibility of protein-bound UTP has not been excluded. By direct assay, no TUT activity was detected in our HF preparations (data not shown).

Fractionation of HF-pretreated RNA on oligo(dT) cellulose. The ability to activate poliovirus RNA by preincubation with HF provided a unique opportunity to study the mechanism of HF action. Since HF-mediated products arise from template self-priming, it was important to examine the 3′ ends of preincubation-activated RNA. Poliovirus RNA was preincubated with HF, reisolated after phenol extraction, and then purified on oligo(dT) cellulose to select for intact molecules. The efficiency of the oligo(dT) cellulose column fractionation was determined by collecting both bound [poly(A)$^+$] and unbound [poly(A)$^-$] RNA and end labeling the 3′ ends with [^{32}P]pCp and RNA ligase. The end-labeled RNA was subjected to nearest-neighbor analysis (Fig. 2). Bound RNA had A at its 3′ end, whereas unbound RNA had a distribution of U, G, A, and C (Fig. 2). The HF-pretreated, fractionated RNAs were also tested for template activity with 3D^{pol} both

TABLE 3. Requirements for activation of poliovirus RNA by HF pretreatment[a]

Reaction conditions	Amt of [^{32}P]UMP incorporated (cpm/50-μl reaction)	% of complete
Complete	122,500	100
−HF	4,900	4
−NTPs[b]	116,375	95
−Mg^{2+}	6,125	5
−$ZnCl_2$	121,500	100
−DTT	89,425	73

[a] The reaction conditions were described in Materials and Methods, and labeled RNA was analyzed as described in Table 1, footnote *a*.
[b] NTPs, Nucleoside triphosphates.

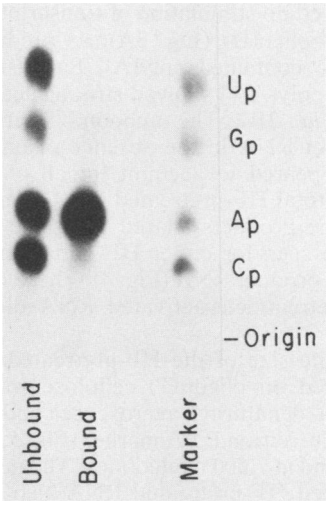

FIG. 2. Nearest-neighbor analysis of end-labeled HF-pretreated RNA after oligo(dT) cellulose fractionation. Preincubation-activated RNA was fractionated on oligo(dT) cellulose into bound [poly(A)$^+$] and unbound [poly(A)$^-$] samples. The RNA was 3′ end labeled with [^{32}P]pCp and subjected to nearest-neighbor analysis, as described in Materials and Methods. Marker nucleoside monophosphates were produced by piperidine hydrolysis of [^{32}P]UMP-labeled RNA from the oligo(U)-primed 3D^{pol} transcription of poliovirus RNA.

in the presence and absence of oligo(U) primer. Activity in the absence of oligo(U) primer indicates HF-pretreatment activation. Transcription of bound [poly(A)$^+$] RNA by 3D^{pol} was stimulated by the presence of oligo(U) (Fig. 3A), yielding products up to 7,500 nucleotides long, as expected (Fig. 4). However, poly(A)$^+$ RNA was not activated by HF pretreatment, i.e., it was not transcribed in the absence of oligo(U) or HF (Fig. 3B), although overexposure of the autoradiogram showed that a very minor amount of product RNA was synthesized (Fig. 4). In contrast, unbound RNA

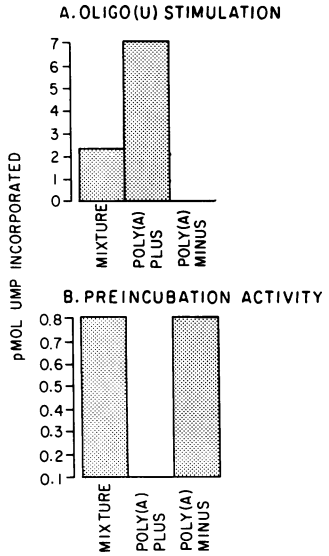

FIG. 3. Transcription stimulation by oligo(U) and activation of HF-pretreated RNA. Preincubated RNA (1 μg) was tested for transcription stimulation by oligo(U) (A) or preincubation activity (B) both before and after fractionation on oligo(dT) cellulose. The reaction conditions were described in Materials and Methods.

[poly(A)$^-$] showed no stimulation of transcription activity in the presence of oligo(U) (Fig. 3A), as predicted, since it should not have contained poly(A). Surprisingly, the unbound fraction [poly(A)$^-$] showed striking activation by HF pretreatment (Fig. 3B). The unbound fraction generated abundant product RNA in the absence of oligo(U) or HF, and, in fact, appeared to account for all of the activated template in the total HF-pretreated RNA sample (Fig. 3B). Furthermore, the products shared the same characteristic size distribution seen for either HF-pretreatment-activated or HF-mediated product RNA (Fig. 4). These results suggest that the HF-pretreatment-activated RNA did not contain poly(A).

To examine the size of the HF-pretreated template, the RNA fractionated on oligo(dT) cellulose was electrophoresed through a denaturing agarose gel and stained with ethidium bromide. Virion RNA marker (Fig. 5, lanes 1 and 5) migrated as a band at 7,500 nucleotides. The major portion of the unfractionated HF-pretreated RNA also migrated as a band at 7,500 nucleotides, although some smaller heterogeneous material was also present (Fig. 5, lane 2). An identical size distribution was seen for the poly(A)$^+$ fraction of the HF-pretreated template (Fig. 5, lane 3). The poly(A)$^-$ fraction of the preincubated RNA was represented by a smear of material from approximately 2,000 nucleotides to what appears to be full-length template (Fig. 5, lane 4). It must be noted that the gel system used is not capable of resolving

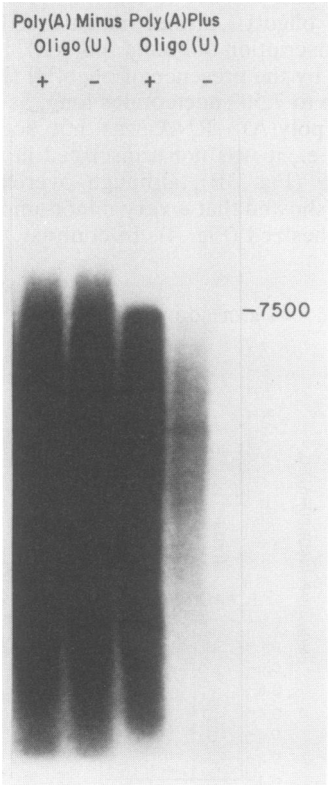

FIG. 4. Denaturing agarose gel analysis of transcripts of HF-pretreated RNA templates fractionated on oligo(dT) cellulose. Poly(A)$^+$ [oligo(dT) bound] and poly(A)$^-$ [oligo(dT) unbound] RNAs (1 μg) were transcribed by 3D^{pol} in either the presence or absence of oligo(U). Product RNA labeled with [^{32}P]UTP was synthesized under standard reaction conditions, extracted with phenol, and precipitated with ethanol before analysis by denaturing agarose gel electrophoresis.

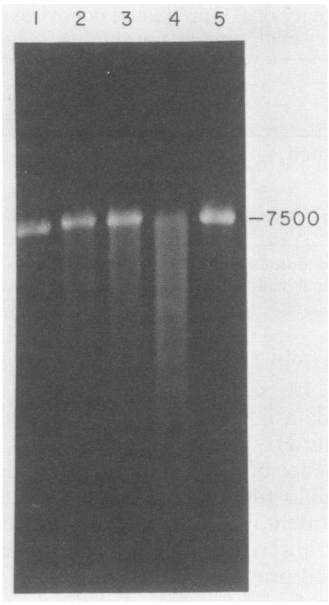

FIG. 5. Denaturing agarose gel analysis of HF-pretreated RNA before and after fractionation on oligo(dT) cellulose. HF-pretreated RNA was fractionated on oligo(dT) cellulose as described in Materials and Methods. The RNA was then analyzed by electrophoresis in CH_3HgOH-agarose gels and stained with ethidium bromide. Approximately 500 ng each of nonfractionated HF-pretreated RNA (lane 2), poly(A)$^+$ HF-pretreated RNA (lane 3), poly(A)$^-$ HF-pretreated RNA (lane 4), and virion RNA marker (lanes 1 and 5) was used.

full-length molecules that differ only in the presence or absence of a poly(A) tract (50 to 100 nucleotides) (1, 38). However, the prominent full-length band present in the poly(A)$^+$ sample (Fig. 5, lane 3) was absent in the poly(A)$^-$ material (Fig. 5, lane 4).

The data presented above demonstrate that the HF-pretreatment-activated RNA did not contain a normal 3′ poly(A) tract. In an effort to determine whether the HF-pretreated RNA was enriched for 3′ ends containing a specific sequence derived from a region near the original 3′ end, which would indicate a preferred cleavage site by the HF nuclease, an S1 nuclease protection experiment was performed on cloned poliovirus cDNA hybridized to HF-pretreated RNA. No detectable enrichment for specific 3′ ends coming from within the 3′ 455 nucleotides of the poliovirus cDNA was found (data not shown). This suggests that the nucleolytic cleavage which occurred in the preincubation reaction was random.

Size selection of HF-pretreated RNA. The data presented above strongly support the interpretation that the HF reaction that activates poliovirus RNA template results in removal of the poly(A) tract from its 3′ end. However, the possibility was considered that the poly(A) tract was still present, yet unable to interact with oligo(dT) cellulose. For example, extensive base pairing with covalently attached poly(U) added to the poly(A) by the putative TUT might conceivably prevent interaction with the column resin. To examine this possibility, HF-pretreated RNA was size selected and purified from preparative methyl mercury hydroxide-containing agarose gels. Full-length HF-pretreated RNA was then end labeled with [^{32}P]pCp and RNA ligase, and a nearest-neighbor analysis was performed. If no modification of the 3′ end was present, then A should have been the only

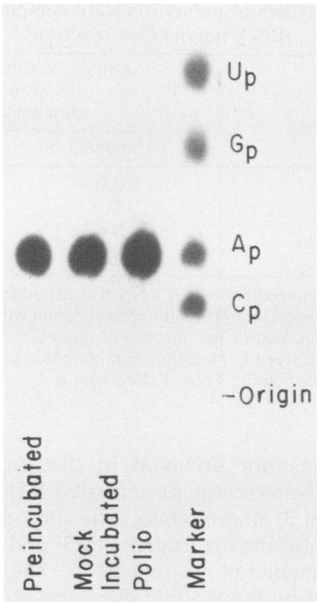

FIG. 6. End analysis of size-selected, HF-pretreated and mock-HF-pretreated RNA. Poliovirus RNA was preincubated in the presence or absence of HF, as described in Materials and Methods. The RNA was then extracted with phenol and precipitated with ethanol before preparative denaturing agarose gel electrophoresis. Full-length RNA was isolated from the gel, end labeled with [^{32}P]pCp, and subjected to nearest-neighbor analysis, as described in Materials and Methods. Poliovirus RNA was used as a control, and the marker nucleoside monophosphates were prepared as described in the legend to Fig. 2.

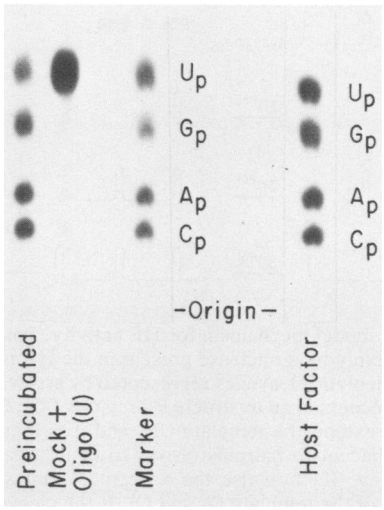

FIG. 7. End analysis of HF-pretreated and HF-mediated templates by using the poliovirus polymerase. HF-pretreated template was labeled with [^{32}P]UTP by using the poliovirus polymerase, as described in Materials and Methods. In a similar experiment poliovirus RNA was end labeled in a standard reaction mixture containing HF and 3D^{pol}, except that [^{32}P]UTP was the only nucleotide present. The labeled RNA was subjected to nearest-neighbor analysis, as described in Materials and Methods.

labeled nucleotide. The full-length HF-pretreated RNA had A at its 3′ end (Fig. 6). This size-selected RNA showed no activation, i.e., it was not transcribed by 3D^{pol} in the absence of oligo(U) or HF. These data support the hypothesis that RNA activated by HF pretreatment does not contain poly(A) and is less than genome length, whereas HF-pretreated RNA of genome length still has poly(A) and is not activated by HF pretreatment.

Labeling of 3′ ends of templates activated by HF pretreatment with 3D^{pol}. To specifically examine the 3′ ends of RNA templates activated by HF pretreatment, 3D^{pol} was used to transcribe HF-pretreated RNA in the presence of [α-^{32}P]UTP but in the absence of any other ribonucleoside triphosphates. Only activated templates are transcribed by 3D^{pol} in the absence of primer or HF. In this way, the template molecules actually being utilized by 3D^{pol} can be analyzed specifically, rather than the total population of molecules. The results of this experiment are given in Fig. 7 and Table 4. HF-pretreated template was incubated with [α-^{32}P]UTP and 3D^{pol}, isolated by gel exclusion on Sephadex G-50, and then subjected to alkaline hydrolysis followed by high-voltage paper electrophoresis. The HF-pretreated RNA received UMP incorporated as a 3′ nearest neighbor, with an equal distribution of all four nucleoside monophosphates (Fig. 7). This shows that the active elongating 3′ ends of the HF-pretreated templates were heterogeneous, and it also indicates that there was little, if any, transcription of a poly(A) tract, since transcription of poly(A) would have been expected to yield a majority of UMP residues adjacent to other UMP residues. Mock-HF-pretreated RNA transcribed with 3D^{pol} and oligo(U) primer in the presence of [α-^{32}P]UTP was also analyzed as a control. This sample yielded a majority of UMP neighbors, as expected (Fig. 7 and Table 4). In analogous experiments, HF-pretreated RNA also stimulated the incorporation of either CTP or ATP in the absence of the other three ribonucleotides 5- to 10-fold over that by the mock-incubated controls (data not shown); however, for unknown reasons, labeling with UTP was more efficient than with other nucleotides. Taken together these results are in agreement with the oligo(dT) cellulose fractionation data which imply that the templates activated by HF treatment lacked poly(A) and utilized heterogeneous 3′ ends for elongation by 3D^{pol}.

In a similar experiment poliovirus RNA that had not been pretreated with HF was used in a reaction mixture containing both HF and 3D^{pol} but with [α-^{32}P]UTP as the only nucleotide present. Nearest-neighbor analysis (Fig. 7) yielded results identical to those for the HF-pretreated template (Fig. 7), implying that the two reactions likely proceeded by the same mechanism.

Oxidation of 3′ ends. It has been shown that HF-mediated products are covalently linked to the template by a self-priming reaction (40). It is likely that HF-pretreated template products arise in the same way. The data presented above

TABLE 4. Nearest-neighbor analysis of 3′ ends of mock- and HF-pretreated RNA templates[a]

Marker comigration	Amt (%) of ^{32}P recovered (cpm)	
	HF pretreated	Mock HF pretreated + oligo(U)
UMP	2,619 (22)	9,843 (83)
GMP	3,148 (27)	826 (7)
AMP	2,690 (23)	533 (4)
CMP	2,982 (26)	462 (4)
Origin	94 (1)	93 (1)

[a] Nearest-neighbor analysis was performed as described in Materials and Methods. An autoradiograph of the chromatogram was used to locate areas of radioactivity, which were then cut from the paper and quantitated by liquid scintillation spectroscopy.

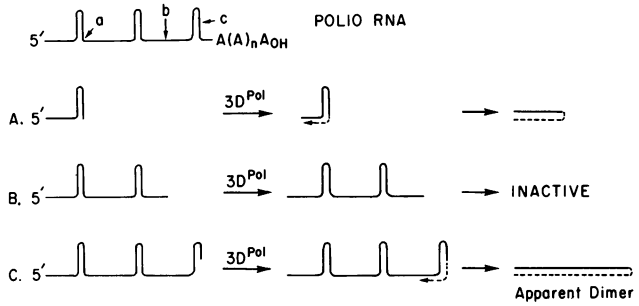

FIG. 8. A model mechanism for HF activity. Poliovirus RNA is cleaved randomly by a nuclease present in the HF preparation (top diagram; nucleolytic cleavages represented by arrows a, b, and c). If the cleavage occurs in an unstructured region of the RNA (arrow b), $3D^{pol}$ cannot extend the template (B), and it is therefore inactive. Cleavages adjacent to hairpins (arrow a) or in a base-paired stem (arrow c) allow $3D^{pol}$ to use the nascent 3' end as a primer for transcription of the template (A and C). If the cleavage occurs in a hairpin near the 3' teminus (arrow c), extension by $3D^{pol}$ results in an apparent dimer (C).

are consistent with this hypothesis except that it does not appear to be the normal 3' poly(A) end of the template that is elongated. Rather, heterogeneous 3' ends, likely arising from a nucleolytic activity, are the active primers for product addition. A possible model for such a mechanism is given in Fig. 8 and is discussed below. To test this hypothesis, poliovirus RNA was oxidized under conditions in which the 3' ribose *cis* diol is converted to a dialdehyde. A dialdehyde on the ultimate 3' residue prevents further nucleotide addition to the template molecule. If, however, other priming 3' ends are generated during the HF or HF-pretreatment reactions, then the use of the oxidized template should have no effect on these reactions.

Poliovirus RNA was oxidized by the method of Marsh and Pace (25). The efficiency of oxidation was monitored by the reduced ability of the RNA to accept [^{32}P]pCp in the presence of RNA ligase. More than 99% of the RNA molecules were oxidized (Table 5). The oxidized RNA was examined by denaturing agarose gel electrophoresis and shown to be largely intact (data not shown). The oxidized RNA remained an efficient template for transcription by $3D^{pol}$ in the presence of oligo(U), as expected (Table 6). More importantly, the oxidized RNA was also activated by preincubation with HF. There was no difference in the abilities of oxidized and nonoxidized RNAs either to be transcribed in the presence of HF or to be activated by preincubation with HF. Product RNAs from both oxidized and nonoxidized templates gave identical size distributions on denaturing agarose gels (data not shown). These experiments clearly demonstrated that the original 3' end of the virion RNA was not involved in the formation of HF-mediated or HF-pretreatment-activated RNA products.

TABLE 5. [^{32}P]pCp 3' end labeling of oxidized RNA[a]

Poliovirus RNA template	Amt of [^{32}P]pCp incorporated (cpm)
Unmodified	1.71×10^6
Oxidized	1.52×10^4
Unmodified + oxidized	1.73×10^6

[a] The reaction conditions were described in Materials and Methods. Approximately 650 ng of either unmodified or oxidized poliovirus RNA was used in each reaction mixture. The reaction mixture containing both RNA preparations had a total of 1.3 μg of RNA; the reaction was done to ensure that the oxidized RNA did not inhibit RNA ligase. Labeled product RNA was analyzed as described in Table 1, footnote *a*.

TABLE 6. Activities of poliovirus RNA templates in poliovirus RNA polymerase reaction[a]

Conditions	Amt of [α-^{32}P]UMP incorporated (pmol/50-μl reaction) with poliovirus RNA:	
	Untreated	Oxidized
No primer	<0.05	<0.05
Oligo(U)	5	6.5
HF	0.6	0.8
HF pretreatment	0.9	1.1

[a] Untreated or oxidized poliovirus RNA was tested for activity with $3D^{pol}$ in the presence of oligo(U) or HF, after preincubation with HF and reisolation by phenol extraction, and in the absence of oligo(U) and HF. The reaction conditions were described in Materials and Methods. Labeled product RNA was analyzed as described in Table 1, Footnote *a*.

HF contains a 3' phosphatase. The data presented above are clear in identifying heterogeneous 3' ends as those active in priming HF-mediated product RNA. The ends were most likely generated by nucleolytic cleavage of the RNA either during pretreatment with HF or simply during the standard HF-$3D^{pol}$ reaction. If this model is correct, then the HF should contain either a nuclease that leaves 3' OH groups that may then be elongated by $3D^{pol}$ or a 3' phosphatase that could remove any 3' phosphate produced by nucleolytic cleavage. The HF was examined for phosphatase activity under standard pretreatment conditions except that the nucleoside triphosphates were removed from the reaction mixture. [^{32}P]pCp was incubated with either HF or a control buffer, spotted onto paper at various times, and subjected to high-voltage paper electrophoresis. [^{32}P]pCp is labeled at the 5' phosphate group only; therefore, if the HF contains a 5' phosphatase activity, then radioactivity would be expected to appear as P_i. If only a 3' phosphatase is present, then the radioactivity shifts from pCp to the pC spot. A nearly quantitative shift from pCp to pC, indicating the presence of a 3' phosphatase, is clearly shown in Fig. 9. A small amount of P_i was also generated, which was likely due to a minor amount of 5' phosphatase. The control sample showed no degradation of pCp.

DISCUSSION

Efforts to understand the biochemistry of poliovirus RNA-dependent RNA synthesis have resulted in the development of in vitro systems that catalyze transcription of poliovirus RNA templates. The essential components consist of the viral polymerase $3D^{pol}$ and an HF fraction from uninfected HeLa cells. When poliovirus RNA is used as the template, the product RNAs are heterogeneous in size, the largest being approximately twice the template length (20, 40), and they contain both nascent plus- and minus-strand sequences (20). The mechanism involved in the generation of these dimer-length products appears to be formation of a 3'-terminal hairpin on the template which serves as primer for product RNA synthesis (40). The resulting product RNA is therefore covalently linked to the template. It is not known whether such template-primed products represent intermediates of the normal replication reaction or whether they are aberrant products of the combined activities present in the in vitro reaction.

Studies described in this report were designed to identify the 3' ends of the elongating self-priming templates. To this

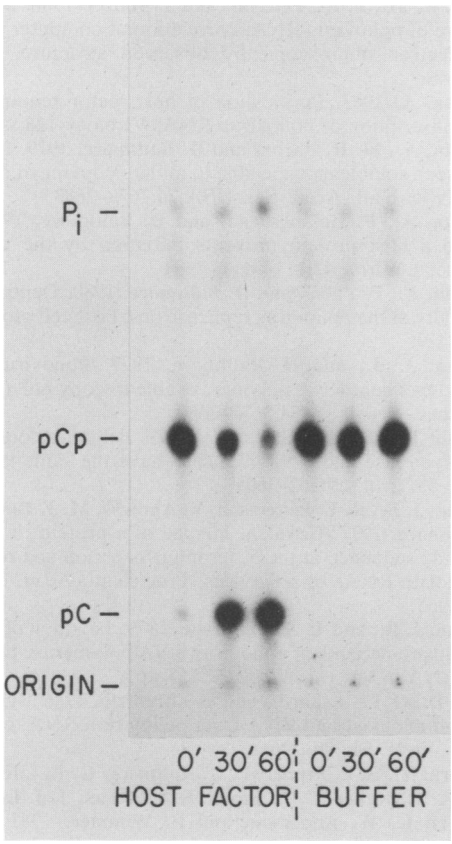

FIG. 9. Phosphatase analysis of HF preparation. [^{32}P]pCp was incubated with HF, as described in Materials and Methods. Samples were spotted onto paper and subjected to high-voltage paper electrophoresis, exactly as described for the nearest-neighbor analysis. The locations of P_i, pCp, and pC were determined by the migration of radioactive markers in adjacent lanes.

end, we first determined that activation of the RNA template by HF could occur before, and independent of, transcription by 3D^{pol}. Preincubation of poliovirus RNA with HF followed by phenol extraction yielded modified RNA that was able to be transcribed by 3D^{pol} without either oligo(U) or HF. This allowed the analysis of HF-modified RNA in the absence of a polymerizing activity. It was shown that the molecules that were activated by HF for subsequent elongation no longer contained their original 3′ poly(A) sequences. Instead, the 3′-terminal nucleotides of elongation templates were shown to contain an approximately equal distribution of each of the four nucleoside monophosphates. In addition, template molecules that were chemically blocked at their original 3′ ends showed no reduction in activity after preincubation with HF or in an HF-mediated polymerase reaction.

The results described above clearly indicate that elongation of the templates in HF-mediated reactions or activated by HF-pretreatment reactions was from heterogeneous 3′ ends, not the original 3′ poly(A) end. We postulate that during either HF pretreatment or an HF-mediated reaction, random nucleolytic cleavages of the template occur. Since poliovirus RNA molecules are capable of forming extensive base-paired secondary structures (11), at least some of the resulting templates would contain 3′-terminal hairpins that could be elongated by 3D^{pol} or reverse transcriptase. A model that describes what we believe are the most likely reactions occurring during HF pretreatment of poliovirus RNA with HF, as well as during the subsequent formation of product RNAs by 3D^{pol}, is depicted in Fig. 8. The generation of products that appear to be of dimer length would arise from elongation of hairpins near the original 3′ end of the template (Fig. 8C). Computer-predicted secondary structures of poliovirus RNA indicate a stable hairpin is present at position 7372 (K. Currey, personal communication). Transcription from this hairpin to the end of the template would yield products that appear to be dimers. Indirect evidence in support of this mechanism comes from the recent observation that 3D^{pol} can extend RNA templates from an internal site (23).

The specificity or identity of the nuclease acting as HF in these reactions is unknown. There was also a 3′ phosphatase activity in the HF preparation. S1 mapping experiments indicated that there were not specific, markedly preferred cleavage sites in the poliovirus RNA but rather, the 3′ ends generated by the HF were heterogeneous and apparently random. Whether there are any preferences for secondary structures (e.g., cutting adjacent to or within hairpin structures) has not been determined. The nuclease activity in the HF preparations was not extensive. After incubation with HF, the vast majority of the template RNA remained intact (Fig. 5, lane 2). Transcription of poliovirus RNA in the presence of HF is relatively inefficient. Considering the number of A residues present in the template and the number of U residues incorporated, we estimate that only a small fraction of input RNA actually serves as template. Given this low efficiency, it is reasonable that a trace-level nuclease is active in the system, generating templates capable of elongation, yet leaving most RNA intact and therefore inactive.

Recently, it was proposed that a TUT acts as HF in vitro (3, 4). Indeed, the addition of uridylate residues to the 3′ poly(A) end of a template could result in a hairpin that upon elongation by 3D^{pol} would yield dimer-length products. However, this reaction would not have been reasonable for the generation of elongation templates in the studies described here. No TUT activity was detectable in our HF preparations. More importantly, 3′-end analysis and chemical oxidation of the 3′ terminal adenylate residue of the template argue strongly against a TUT reaction being involved in template elongation.

Nucleolytic activation of poliovirus RNA templates appeared to be responsible for all of the HF-mediated synthesis that we observed. We have not examined the 3′ ends of other RNA templates. Globin mRNA, for example, will serve as a template with HF and 3D^{pol} (20, 39); in our laboratory the reaction was inefficient compared with that with poliovirus RNA. However, the products show a high percentage of dimer-length RNA. The low efficiency of utilization may simply be due to presentation of relatively few cutting sites; the production of predominantly dimer-length products may reflect either a hypersensitive nucleolytic site near the 3′ end or simply a site that after cleavage forms hairpins better than do other sites.

This report shows that an HF preparation that contained a low-level nuclease (and possibly a 3′ phosphatase) was sufficient to generate apparent dimer-length products, as well as smaller heterogeneous products as previously reported (20). The possibility that the same or similar products could be generated by other HFs has not been excluded; indeed, HF preparations that contain TUT activity may produce some products that are elongated from the original poly(A)$^+$ terminus of the template. Other laboratories have described the production of monomer-length complementary

RNA products from a poliovirus RNA template (8, 12), although the mechanism of initiation by these HF preparations was not determined and was likely clouded by the relative impurity of the polymerase preparations used.

The results presented here have two significant implications. The first is that the HF activity studied in these, and possibly other investigations, likely has little relevance to poliovirus RNA replication in vivo. The second and perhaps more philosophical implication relates to the approach that has been taken to study the in vitro transcription of poliovirus RNA. It was reasonable, after the purification of $3D^{pol}$ and the observation that it represents only an elongation activity, to search for factors that would allow it to synthesize RNA in response to a template. It is clear that several factors can supply this function, including synthetic oligo(U), a nuclease, perhaps TUT, and possibly many other unidentified factors. A critical point in the elucidation of which factors or activities represent the replication reaction occurring in vivo lies in a careful analysis of the products generated by the reconstituted reaction. The major replication product in vivo is plus-sense, full-length RNA linked to VPg at its 5′ end and polyadenylated at its 3′ end. Ideally, an in vitro system would mimic this reaction. Extreme caution should be exercised in considering models of RNA replication derived from the present in vitro systems. The reactions have been very inefficient and have yet to produce an RNA molecule that has been shown to be identical to any RNA produced in vivo.

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