Excess Synthesis of Viral mRNA 5'-Terminal Oligonucleotides by Reovirus Transcriptase*

(Received for publication, December 3, 1980)

Minoru Yamakawa, Yasuhiro Furuichi, Kunio Nakashima,† Alba J. LaFiandra, and Aaron J. Shatkin

From the Roche Institute of Molecular Biology, Nutley, New Jersey 07110

Short oligonucleotides corresponding to the 5'-terminal sequence of reovirus mRNAs were produced in vitro by virion-associated transcriptase activity. Both capped and uncapped oligonucleotides were synthesized in molar excess relative to mRNA. Yields of uncapped oligomers including ppG-C and ppG-C-U were severalfold greater than the homologous capped structures. In partial reaction mixtures that were nonsupportive for mRNA chain elongation, capped oligomer synthesis was increased. Similarly, oligonucleotide formation was differentially resistant in viral preparations that were inactivated with respect to mRNA synthesis by modification of the genome RNA by dimethyl sulfate alklylation or psoralen photoreaction. The results suggest that reovirus mRNA synthesis involves excessive initiation by reiterative transcription of promoter sites by the reovirus polymerase. Only a small fraction of the resulting oligonucleotides are capped and extended to form full length mRNAs during a subsequent elongation step which is apparently mediated by transcriptase molecules that escape the reiterative phase of transcription.

Purified reoviruses contain RNA polymerase and guanylyl and methyl transferases, enzymes that catalyze the synthesis of capped and methylated viral mRNAs (1). Messengers with 5'-terminal m'GpppN are similarly produced by enzymes associated with many other animal RNA and DNA viruses (2). Blocked and methylated cap structures of the same type are also present on the 5' ends of eukaryotic cellular mRNAs and capped oligomers including ppG-C and ppG-C-U were formed when chain elongation was prevented by incubating purified reovirus cores. Although mRNA synthesis required the presence of all four ribonucleoside triphosphates, oligonucleotides corresponding in sequence to mRNA 5' termini were produced in high molar excess in both complete and partial incubation mixtures. Furthermore, oligonucleotide synthesis was relatively resistant to psoralen photoreaction and dimethyl sulfate alklylation, treatments that altered the viral genome RNA and differentially inactivated mRNA production. The results support a model of transcription involving (i) excessive initiation resulting in the synthesis of short oligonucleotides by reiteration of RNA polymerase at promoter sites, (ii) capping of some of these "initiator" oligonucleotides, and (iii) chain elongation and completion of a fraction of the capped oligonucleotides by transcriptase molecules that escape from the reiterative phase. An analogous cycling scheme has been proposed recently for initiation by E. coli RNA polymerase on the lac UV5 promoter (5).

These findings, together with similar observations on insect cytoplasmic polyhedrosis virus (6) and other transcription systems, suggest that reiterative and excessive initiation relative to full length mRNA production may be a general property of transcriptases.

EXPERIMENTAL PROCEDURES

Preparation of Reovirus Cores—The Dearing strain of reovirus type 3 was purified from infected mouse L cells by fluorocarbon extraction and banding in CsCl as described previously (7). Virions were converted to cores by proteolytic digestion at a virus protein concentration of 1 mg/ml in 0.07 M Tris-HCl buffer, pH 8, containing 0.09 M KCl and 0.5 mg/ml of a-chymotrypsin. After 45 min at 42 °C the cores were collected by centrifugation (10 min at 9000 rpm), washed in 0.05 M Tris-HCl (pH 8), 50 mM KCl, and resuspended at 0.5 mg/ml in 0.01 M Tris-HCl (pH 8), containing 1 mM EDTA or 5 mg/ml in 0.05 M 4-(2-hydroxyethyl)-1-piperazinethesulfonylic acid buffer, pH 8, containing 50 mM KCl.

Transcription Reaction Conditions—Incubation mixtures of 0.1 ml contained 0.07 M Tris-HCl buffer (pH 8), 8 mM Mg-acetate, 0.2 mM concentrations of CTP, ATP, and UTP, 0.4 mM GTP, 0.1 mM S-adenosylmethionine (or, where indicated, 10 μM S-adenosyl[Methyl-3H]methionine), 1 mM phosphoenolpyruvate, 5 units of pyruvate kinase, and 30 μg of reovirus cores. a-32P-labeled ribonucleoside triphosphates were included as indicated at a final specific activity of 0.5-2 Ci/mmol. Under these conditions the synthesis of both mRNA and oligonucleotides increased proportionally at 42 °C for at least 4 h. Qualitatively similar results were obtained with 5-fold higher levels of ribonucleoside triphosphates, and the yields of each of the products were correspondingly increased. Cores that were stored at 4 °C or used directly as chymotrypsin digestes of virus yielded the same transcription products by gel analysis.

Polyacrylamide Gel Electrophoresis—Transcription reaction mixtures were diluted 2-fold with H2O, adjusted to 5 mM EDTA at 0 °C, and extracted with an equal volume of water-saturated phenol followed by ether extraction of the aqueous phase. To minimize masking
of short oligonucleotide products by the radioactive precursor, samples were treated as indicated for 1 h at 37°C with calf intestine alkaline phosphatase (5 units/ml) to convert unreacted ribonucleoside triphosphates to P. The digests were lyophilized, dissolved in 20 μl of 0.02 M Tris-borate buffer, pH 8.2, containing 1 mM EDTA, 7 mM urea, and 0.05% each of xylene cyanol and bromphenol blue, and applied to a 20% polyacrylamide slab gel (0.1 × 28 × 37 cm) that had been prepared for 2 h at 800 V in the same buffer system (4). Electrophoresis was at 800 V until the bromphenol blue marker dye had migrated 20 cm (6–7 h). Autoradiographs of the gels were made with preflashed XR-5 film.

Analyses of Oligonucleotides—Oligonucleotides located by autoradiography were extracted from gel slices in H2O and desalted by desorption to DEAE-Sephadex A-25 and elution in NH4HCO3 as described (6). Oligonucleotide samples were analyzed directly for net charge by DEAE-cellulose column chromatography. The resin (0.6 × 24 cm column) was equilibrated with 50 mM Tris-acetate buffer, pH 8, containing 4 mM EDTA, 7 mM urea, and 50 mM NaCl. Samples were loaded in the same solution with RNase A digests of RNA as markers and eluted with a linear gradient of 0 to 400 mM NaCl. Oligonucleotide structures were deduced from analyses by high voltage paper electrophoresis at pH 3.5 or paper chromatography in isobutyric acid, 0.5 M NH4OH (0.65, v/v) following enzymatic digestion under the following conditions (1, 6, 8c): calf intestine alkaline phosphatase (10 units/ml in 0.1 M Tris buffer, pH 8, 37°C, 30 min); RNase T2 (25 units/ml in 25 mM sodium acetate buffer, pH 4.5, 37°C, 60 min); nuclease P, (0.125 mg/ml in 10 mM sodium acetate buffer, pH 6, 37°C, 60 min); and tobacco acid pyrophosphatase (0.06 units/ml in 20 mM sodium acetate buffer, pH 6, containing 20 mM mercaptoethanol, 37°C, 30 min) (9). Samples sequentially treated with two enzymes were exchanged with phenol and ether between the two digestion steps. The migration positions of digestion products were compared by paper electrophoresis with marker compounds GpC, ppGp, and the four ribonucleoside monophosphates and by paper chromatography with GpppG, mGpppG, mGpppG, and pC.

Photoreaction of Cores—Viral cores suspended at 250 μg/ml in 10 mM Tris-HCl, pH 8, and 1 mM EDTA were irradiated at λ<sub>265 nm</sub> in the presence of 4-aminomethyl-4,5,8-trimethylpsoralen or angelicin (kindly provided by Prof. G. Rodighiero, Instituto di Chimica Farmaceutica dell'Università, Padova, Italy) as described in detail (10, 11). Cores were diluted into transcriptase reaction mixtures for assay. To test for cross-linking of the viral genome, RNA was phenol-extracted from photoreacted cores, exposed to denaturing conditions in dimethyl sulfoxide, and analyzed by electrophoresis in 1.7% agarose gels with ethidium bromide staining, all as described previously (11).

Dimethyl Sulfate Alkylation—Virus cores (25 μg) in 0.1 ml 0.05 M phosphate buffer pH 7 were treated with dimethyl sulfate at room temperature. Samples were diluted with 20 volumes of 0.05 M Tris-HCl, pH 8, containing 0.15 M KCl, and the cores were pelleted, resuspended in a 0.02 ml of 0.01 M cacodylate buffer, pH 8, containing 0.06 M sucrose, deoxycholate, sonicated briefly, and tested for RNA polymerase activity. mRNA products were analyzed by sedimentation in 5–20% glycerol gradients (7). Reaction conditions for assaying other virus-associated enzyme activities including nucleotide phosphohydrolase, guanylyltransferase, and methyltransferases were as reported previously (11).

Materials—S-<sup>32</sup>P-labeled ribonucleoside triphosphates were purchased from Amersham Corp. and S-adenosyl-[Me-<sup>32</sup>W)methionine (specific activity, 70 Ci/mmol) was from New England Nuclear. AMF<sup>W</sup> was from Calbiochem and dimethyl sulfate was from Mathieson, Coleman & Bell, Inc. Marker compounds included GpC, ppGp, GpppG, mGpppG, and mGpppG from P-L Biochemicals, Inc. Calf intestine alkaline phosphatase, RNase T2, and nuclease P1 were obtained from Boehringer-Mannheim, Calbiochem, and Yamasa Shoyu Co., respectively. Tobacco pyrophosphatase was kindly provided by Drs. M. Miwa and T. Sugimura, National Cancer Center Research Institute, Tokyo, Japan.

RESULTS

Detection of Reovirus Oligonucleotides Produced in Vitro during mRNA Formation—A variety of different types of animal viruses including human reoviruses contain transcriptase activity associated with the viral genome (12). In cells infected with these viruses the replicative cycle begins with viral mRNA synthesis directly from parental virions, i.e., without the need to form a new virus-specific RNA polymerase. Purified reovirions, converted to viral cores by chymotrypsin digestion, contain transcriptase and other enzymes involved in the formation of mRNA 5' terminal caps (1). The virion-associated activities are in a highly stable configuration, permitting correct initiation and synthesis of functional reovirus mRNAs for extended periods in vitro. To determine if the reovirus enzymes also produce prematurely terminated transcripts, possibly related to the oligonucleotides found in purified virions (13), transcription products labeled with [α-<sup>32</sup>P]-CTP were analyzed by electrophoresis in RNA sequencing gels. The high molecular weight RNAs remained near the top of the gel, and no intermediate length products were apparent as bands between the origin and the marker dyes, xylene cyanol and bromphenol blue (Fig. 1, lane 2). However, several distinct bands (Fig. 1, the prominent bands designated 1–6) were obtained in the region between the bromphenol blue dye and the bottom of the gel. All but the spot in the position of bromphenol blue dye appeared to be products of incubation and were absent from reaction mixtures that were kept at 0°C (Fig. 1, lanes 1 and 3). When an aliquot of the same products was digested with alkaline phosphatase before

---

**Fig. 1.** Polyacrylamide gel electrophoresis of reovirus mRNA and oligonucleotide products. Duplicate transcription reaction mixtures were made with [α-<sup>32</sup>P]-CTP (specific activity, 0.5 Ci/mmol) as described under "Experimental Procedures." One sample was kept at 0°C as control and the other was incubated at 42°C for 3 h. They were then extracted with phenol followed by ether, and one-half of each was treated with phosphatase before electrophoresis in 20% polyacrylamide gels. Lanes 1 and 3, 0°C control before and after phosphatase; lanes 2 and 4, 42°C incubation products before and after phosphatase treatment, respectively. XC, xylene cyanol dye; BPB, bromphenol blue dye.
gel electrophoresis, the pattern shown in Fig. 1, lane 4, was obtained. Most of the radioactive precursor was converted to P, which migrated off the gel. Spots 1 and 2 and a poorly resolved spot 3 disappeared and were replaced by three more slowly migrating spots: 7, 8, and 9. By contrast, spots 4, 5, and 6 remained essentially unaffected and migrated in the same relative positions after exposure to phosphatase (Fig. 1, lane 4: 4', 5', and 6'). The following analyses indicate that these products include mainly uncapped (phosphatase-sensitive) and smaller amounts of capped (phosphatase-resistant) oligonucleotides corresponding to the 5'-terminal nucleotide sequence of reovirus mRNAs.

Characterization of Oligonucleotides—The 5' ends of the multiple species of reovirus mRNA have the common sequence, m'GppG U-A . . . (14). To determine if the short transcription products detected by gel electrophoresis were related to this mRNA initiation sequence, the individual bands in Fig. 1 were eluted from the gel, desalted, and further characterized. Spot 1 had a net charge of about -3.5 as determined by DEAE-cellulose, 7M urea column chromatography (Fig. 2A). It migrated by paper electrophoresis at pH 3.5 as a single component (Fig. 3A, lane 1) that shifted to the position of authentic G-C after phosphatase treatment (Fig. 3A, lane 2). Digestion with RNase T2 converted the radioactivity in spot 1 to material migrating with marker ppGp (Fig. 3A, lane 3) while sequential treatment with phosphatase followed by RNase T2 yielded Gp (Fig. 3A, lane 4). Finally, all the radioactivity was released as P by exposure to P1 nuclease (Fig. 3A, lane 5). The results demonstrate that spot 1 is ppG-C which corresponds to the uncapped initial dinucleotide of viral mRNAs. Spot 9 from the sample treated with phosphatase before gel electrophoresis also co-migrated with G-C (Fig. 3A, lane 6), consistent with its derivation from ppG-C (spot 1) by removal of the unblocked 5'-phosphates. The same oligonucleotide was also synthesized by viral cores as the predominant product in incomplete reaction mixtures containing only GTP and CTP, confirming the structure ppG-C (see Fig. 4A below).

Oligonucleotide 2 is apparently one nucleotide longer than ppG-C and had a net negative charge of about -4 by column chromatography (Fig. 2A). Like spot 1, it migrated as a single

![Fig. 2. DEAE-cellulose column chromatography of oligonucleotides.](image)

FIG. 2. DEAE-cellulose column chromatography of oligonucleotides. Net negative charges of the low molecular weight products in Fig. 1 were determined by chromatography on Whatman DE52 with RNase A digests of tRNA as markers (1). Each sample was analyzed separately, but the profiles are plotted together for convenience.

![Fig. 3. High voltage paper electrophoresis of oligonucleotides and their enzymatic digestion products.](image)

FIG. 3. High voltage paper electrophoresis of oligonucleotides and their enzymatic digestion products. Oligonucleotide products were synthesized in the presence of [α-32P]CTP, except for samples in lanes 5-7 of B which were labeled with [α-32P]UTP. Purified, desalted oligonucleotides were digested as described under "Experimental Procedures" and analyzed by paper electrophoresis at pH 3.5 with marker 3'-mononucleotides, ppGp, GpC, and P1. A: spot 1 without treatment (lane 1) or digested with calf phosphatase (lane 2), RNase T2 (lane 3), phosphatase followed by T2 (lane 4), or P1 nuclease (lane 5). Lane 6 is spot 9 without enzymatic digestion. B: spot 2 untreated (lane 1) or digested with RNase T2 (lane 2) or phosphatase (lane 3), spot 8 untreated (lanes 4 and 5) or digested with P1 nuclease (lane 6) or RNase T2 (lane 7). C: spot 4 untreated (lane 1) or digested with tobacco pyrophosphatase followed by phosphatase (lane 2); spot 5 untreated (lane 3) or following tobacco pyrophosphatase and phosphatase treatment (lane 4).

![Fig. 4. Product analyses of complete and partial transcription mixtures.](image)

FIG. 4. Product analyses of complete and partial transcription mixtures. Reaction mixtures containing the indicated α-32P-labeled ribonucleoside triphosphate were incubated for 3 h at 42°C (or kept at 0°C where indicated), and the products were digested with calf phosphatase and analyzed by gel electrophoresis as described under "Experimental Procedures." A, [α-32P]CTP: lane 1, control (CON) without viral cores (0°C); lane 2, control without cores; lane 3, control with cores (0°C); lane 4, complete [COMP]; lane 5, minus ATP; lane 6, minus ATP and UTP (−A,UTP). B, [α-32P]UTP: lane 1, control without cores; lane 2, complete; lane 3, minus ATP. C, [α-32P]ATP: lane 1, without cores; lane 2, complete. D, [α-32P]GTP: lane 1, control without cores; lane 2, complete; lane 3, minus ATP; lane 4, minus ATP and UTP.
main component by paper electrophoresis (Fig. 3B, lane 1) and yielded ppGp by RNase T2 digestion (Fig. 3B, lane 2). These results indicate the presence in spot 2 of 5'-terminal G-C in a structure ppG-C-N. Phosphatase treatment of spot 2 converted it to a more slowly migrating spot (Fig. 3B, lane 3) that moved with spot 8 (Fig. 3B, lane 4), suggesting that the two are ppG-C-N and G-C-N, respectively. Spot 8 was also labeled with [a-32P]UTP (Fig. 3B, lane 5) and yielded pU by P, nuclease treatment (Fig. 3B, lane 6) and Cp upon RNase T2 digestion (Fig. 3B, lane 7), establishing N as U. The same results were obtained for spot 2 with [32P]UTP (data not shown). In agreement with these findings, spots 2 and 8 were not produced in reaction mixtures containing GTP and [a-32P]GTP but no UTP (Fig. 4A). From these data it can be concluded that spots 1 and 2 are uncapped mRNA 5'-terminal sequences, ppG-C and ppG-C-U, that are converted by phosphatase treatment to the corresponding G-C (spot 9) and G-C-U (spot 8). Consistent with these structures, spots 9 and 8 had net charges of -1 and -2 (Fig. 2B), and each yielded Gp and Cp as the exclusive radioactive products of RNase T2 and nuclease P, digestion, respectively (data not shown). Spots 3 and 7 are probably ppG-C-U-A and G-C-U-A, respectively, since their synthesis required all four rNTPs, and they were labeled in mixtures containing [a-32P]ATP while the other oligonucleotides were not (Fig. 4C). In agreement with this sequence, RNase T2 digestion of [32P]ATP-labeled spot 7 yielded mainly (90%) Up by nearest neighbor transfer (data not shown).

The bands designated 4-6 in Fig. 1 were unchanged by phosphatase treatment and had net negative charges of about -3.5, -4.4, and -5.3, respectively (Fig. 2C). They were sensitive to tobacco pyrophosphatase, an enzyme that cleaves 5'-terminal structures such as GpppG and m'GpppG (9). As shown in Fig. 3C, treatment with the tobacco enzyme followed by phosphatase converted spot 4 to G-C (Fig. 3C, lanes 1 and 2) and spot 5 to material migrating in the position of G-C-U (Fig. 3C, lanes 3 and 4). Thus, spots 4 and 5 are the 5'-blocked counterparts of uncapped oligonucleotides G-C and G-C-U, and spot 6 presumably is capped G-C-U-A.

To determine the 5'-terminal structures of the capped oligonucleotides, products synthesized in complete transcription reaction mixtures containing [a-32P]CTP and S-adenosyl[Me-3H]methionine were separated by gel electrophoresis. After purification, the oligonucleotides were treated with P, nuclease, and the digests were analyzed by paper chromatography. The 32P-labeled material in the digest migrated in the position of pC. Each sample also yielded two 3'H-methylated labeled products that were identified as m'GpppG and m'GpppG". Zarbl et al. (15) also recently reported the formation of m'G-capped oligonucleotides by reovirus cores incubated in complete transcription mixtures with S-adenosyl[Me-3H]methionine as the radioactive precursor. However, we found that the methylated oligomers comprise only a small fraction (2-3%) of the total oligonucleotides. From the ratio of 3'H to 32P it was determined that the capped oligonucleotides also included unmethylated 5'-GpppG. The relative amounts of GppppG, m'GpppG, and m'GpppG" calculated on the basis of the specific activities of 3'H- and 32P-labeled precursors were: 89, 6, and 5% for capped G-C; 60, 16, and 24% for capped G-C-U; and 49, 20, and 31% for capped G-C-U-A, respectively. Note that the extent of methylation increased with oligonucleotide chain length.

The unusually slow migration of G-C (spot 9) and G-C-U (spot 8) relative to the marker dyes is noteworthy. In other studies bromphenol blue and 5'- or 3'-phosphorylated oligonucleotides of chain length 8-10 were found to co-migrate in the same gel system (4, 6). Apparently the high mass/charge ratio of G-C as compared for example to ppG-C (or pG-C and G-Cp) accounts for the aberrant migration of the dimucleoside monophosphate. Similar results were obtained with A-G and other related structures (6).

**Quantitation of Oligonucleotides in Complete and Partial Reaction Mixtures**—The sequencing gel method revealed the presence of both capped and uncapped reovirus oligonucleotides in transcription reaction mixtures incubated under conditions that supported viral mRNA synthesis. To determine if similar oligomers were formed in the absence of chain elongation, products from partial and complete reaction mixtures were compared. Mixtures were exhaustively digested with phosphatase before gel analysis in order to convert the labeled ribonucleoside triphosphate precursor to P. This treatment resulted in some degradation of the high molecular weight RNAs in the complete mixtures but prevented potential masking of oligonucleotide products in the autoradiogram by the radioactive precursor. Control reaction mixtures kept at 0 °C either without addition of virus cores (Fig. 4A, lane 1) or in the presence of cores (Fig. 4A, lane 3) each yielded a spot co-migrating with bromphenol blue that was increased in mixtures incubated at 42 °C without cores (Fig. 4A, lane 2). Since this result indicated that this spot was formed by a chemical reaction not catalyzed by cores, the material was not further analyzed. Complete transcription mixtures incubated at 42 °C yielded RNA, m'Gppp-capped oligonucleotides G-C, G-C-U, and G-C-U-A, and the corresponding uncapped products (Fig. 4A, lane 4). In partial reaction mixtures without ATP the products also included capped and uncapped G-C and G-C-U but not G-C-U-A sequences (Fig. 4A, lane 5). The yields of uncapped G-C and G-C-U were similar to those obtained in the complete reactions while the corresponding capped oligonucleotides were increased 3- to 4-fold in the

**TABLE 1**

Oligonucleotide yields from complete and partial transcription reactions

| Oligonucleotide | [32P]CTP | [32P]UTP | [32P]GTP | [32P]ATP, complete |
|-----------------|---------|---------|---------|-------------------|
| Uncapped        | Complete -ATP | ATP, UTP | Complete -ATP | ATP, UTP |
| G-C             | 40.1    | 41.7    | 11.9    | 22.3             |
| G-C-U          | 21.7    | 28.9    | 73.0    | 166.2            |
| G-C-U-A        | 8.3     | 10.8    |    -    |         |
| Capped          | Complete -ATP | ATP, UTP | Complete -ATP | ATP, UTP |
| G-C             | 2.3     | 6.2     | 22.3    | 2.8              |
| G-C-U          | 2.7     | 9.6     | 37.2    | 3.3              |
| G-C-U-A        | 1.4     | 2.8     | 1.4     | 1.0              |

The values are averages of 3 experiments in which reaction products formed during a 3-h incubation were analyzed by electrophoresis as in Fig. 4. Excised bands were counted by Cerenkov radiation. mRNA yields with [32P]-labeled precursor CTP, UTP, GTP, and ATP were 0.20, 0.28, 0.29, and 0.20 pmol, respectively using an average chain length of 1,500.
partial incubation mixtures (Table I). In incomplete transcription mixtures containing only GTP and [α-32P]CTP synthesis of capped G-C was increased 10-fold (Fig. 4A, lane 6; Table I). The finding that conditions precluding elongation also enhanced capped oligonucleotide formation is similar to the report (5) that initiator oligonucleotide synthesis by E. coli RNA polymerase was increased by omission of 1 or 2 ribonucleoside triphosphates.

To confirm the nature of the various reovirus oligonucleotides labeled with [α-32P]CTP, reactions were also carried out with each of the other α-[32P]labeled rNTPs. As shown in Fig. 4B, complete reaction mixtures containing [32P]CTP yielded oligonucleotides in the positions of G-C-U and G-C-U-A (lane 2). In the absence of ATP the yield of capped G-C-U increased severalfold as compared to <2-fold for uncapped G-C-U (Fig. 4B, lane 3; Table I). The products formed with [α-32P]ATP and three unlabeled rNTPs included uncapped G-C-U-A and a low level of material migrating in the position of the corresponding capped oligonucleotide (Fig. 4C). These results indicate that capped tri- and tetranucleotides are more effectively elongated to form mRNAs than their uncapped counterparts synthesized in complete transcription mixtures. With [α-32P]GTP as precursor in the complete reaction mixture, RNA and capped oligonucleotides were detectable, but no radioactive spots were observed in the positions of uncapped oligomers because phosphatase treatment removed the [32P]-labeled unblocked 5' phosphates (Fig. 4D, lane 2). In the absence of ATP RNA was not formed, and the amounts of capped G-C and G-C-U were increased relative to complete reactions (Fig. 4D, lane 3; Table I). Again capped G-C was markedly increased in the reaction mixture that lacked both ATP and UTP (Fig. 4D, lane 4; Table I).

Effects of Psoralen Photoreaction on Transcription—Irradiation of reovirus cores in the presence of the 4'-substituted psoralen derivative, AMT, results in the formation of psoralen monoadducts and subsequent cross-links in the double-stranded genome RNA (10, 11, 16). As a consequence of template alteration the core-associated RNA polymerase activity is diminished. Cores partially inactivated by psoralen photoreaction produce decreased yields of full length mRNAs, without an apparent rise in prematurely terminated transcripts. This all-or-none effect on mRNA formation suggested that promoter proximal sites in the template RNA might be differentially sensitive to psoralen modification, causing a total block in transcription. Alternatively, if initiation and chain elongation are separable events (as suggested from the results in Fig. 4), synthesis of oligonucleotides corresponding to mRNA initiation sequences would be expected to continue in cores that contain modified template RNAs and are dam-

![Fig. 5. Effect of AMT photoreaction on the formation of mRNA and oligonucleotides.](image-url)

![Fig. 6. Angelicin photoreaction effects on reovirus genome RNA structure and mRNA synthesis.](image-url)
aged with respect to RNA chain elongation. To test these possibilities, AMT photoreacted viral cores were incubated in a complete transcription reaction mixture containing [$\alpha$-$^{32}$P]CTP. The reaction products were compared by gel electrophoresis with those of untreated particles. As shown in Fig. 5, untreated viral cores synthesized 5'-terminal oligonucleotides in high molar excess (5- to 100-fold) relative to full length mRNAs. Furthermore, uncapped oligonucleotides were produced in considerably greater yields than the corresponding capped products. Synthesis of both capped and uncapped oligonucleotides was relatively resistant to AMT photoreaction. For example, mRNA synthesis was diminished by 94% in cores photoreacted for 30 s at 1.2 x 10^{-5} M AMT. Under the same conditions synthesis of the oligonucleotides was decreased by less than 2-fold, indicating that a psoralen-sensitive stage of transcription occurs between reiterative initiation and elongation.

Angelcin is a monofunctional psoralen derivative that also forms pyrimidine photodadducts but, in contrast to AMT, no cross-links in nucleic acids (17). This property was confirmed with reovirus genome RNA. Gel electrophoresis of the double-stranded RNA separated the three size classes of genome segments (Fig. 6A, lane 1-). RNAs that were denatured by loading the sample in the presence of dimethyl sulfoxide migrated more rapidly without resolution of the size classes of single-stranded RNAs (Fig. 6A, lane 1+). The double-stranded RNA remained denaturable after photoreaction with angelcin either in situ (Fig. 6A, lane 2) or after phenol extraction from treated cores (Fig. 6A, lane 3). By contrast, AMT photoreacted RNA was cross-linked and not denatured by exposure to dimethyl sulfoxide (Fig. 6A, lane 4). Although angelcin photoreaction did not cause cross-linking, it diminished RNA synthesis in a time and concentration-dependent manner (Fig. 6B). Thus, the presence of single strand modifications was sufficient to diminish the template activity of reovirus genome RNA for mRNA synthesis by the virion-associated RNA polymerase. Initiation of reovirus transcription as measured by oligonucleotide formation was partially resistant to angelcin photoreaction. For example, irradiation for 7.5 min in the presence of 3.7 x 10^{-4} M angelcin reduced RNA synthesis by 70%, reduced G-C formation by 26%, and reduced capped oligomers not at all (Fig. 7).

**Fig. 7.** Effect of angelcin photoreaction on reovirus mRNA and oligonucleotide synthesis. Reovirus cores were irradiated in 3.7 x 10^{-4} M angelcin and tested for mRNA and oligonucleotide formation as described for Fig. 5.

Diffrential inactivation of reovirus mRNA synthesis by dimethyl sulfate alkylation. A, viral cores were treated for 1 h as described under "Experimental Procedures," and RNA synthesis was then measured by incorporation of $^{32}$P into acid-insoluble material by incubation for 30 min at 42°C in transcription reaction mixtures containing [$\alpha$-$^{32}$P]UTP as radioactive precursor. Arrow indicates 50% inactivation at 10 mM DMS. B, reovirus-associated enzyme activities were tested as described previously (11) using cores treated for the indicated times with 10 mM DMS. Activities: O, RNA polymerase; □, nucleotide phosphohydrolase; △, guanylyltransferase; and ×, methyltransferase.

**Fig. 8.** Differential inactivation of reovirus mRNA synthesis by dimethyl sulfate-treated viral cores—Incubation of reovirus cores with dimethyl sulfate alkylates the genome RNA predominantly at the N7 position of guanosines and also to a small extent the N1 of adenosines (18). DMS treatment of viral cores diminished their capacity for mRNA synthesis. Incubation for 1 h in 10 mM DMS decreased RNA polymerase activity by 50% as measured by incorporation of radioactive nucleotides into acid-precipitable material, and higher concentrations were more inhibitory (Fig. 8A). Other core-associated enzyme activities including the nucleotide phosphohydrolase and guanylyltransferase were resistant to treatment with 10 mM DMS for 3 h or longer (Fig. 8B). The transferase(s) that catalyzes methyl group transfer from S-adenosylmethionine to RNA 5' termini was partially inactivated (30-40%) by exposure to 10 mM DMS for 2-3 h (Fig. 8B).

The transcription products of viral cores partially inactivated by DMS alkylation were examined by both glycerol gradient sedimentation and polyacrylamide gel electrophoresis to determine if alkylation of the template RNA resulted in the synthesis of prematurely terminated transcripts. Full length large, medium, and small species of reovirus RNA were produced by DMS-treated core preparations that contained...
RNA synthesis (Fig. 10A). For example, 1 h treatment at a level of 20 mM DMS essentially abolished RNA synthesis (Fig. 10A), while the yields of uncapped and capped oligonucleotides were affected slightly or not at all (Fig. 10B).

**DISCUSSION**

The RNA polymerase associated with human reoviruses and other double-stranded RNA viruses is tightly associated with the viral genome segments. These duplex templates are transcribed by the polymerase in a conservative manner to form single-stranded, full length copies of the genome RNA plus strands (19). The transcripts function during virus replication as messengers for directing virus-specific protein synthesis and as templates for a replicase activity that produces new genome RNA (20).

Previous studies with reovirus photoreacted with the 4′-aminomethyl derivative of trioxalene showed that covalent modification of the genome RNA resulted in a loss of infectivity and of template activity for RNA synthesis by the virion transcriptase (10). The effect of psoralen photoinactivation on RNA formation appeared to be all-or-none. That is, full length transcripts were synthesized without an increase in prematurely terminated products even in a short incubation period sufficient to allow only one cycle of reovirus transcription or in preparations that were modified to the extent of ~1 psoralen/100 base pairs and retained only a few percent of the original RNA synthesizing capacity (11). It was suggested that the polymerase might be unable to initiate on templates that were structurally altered by attachment of psoralens at random sites along the chains. The results of the present study eliminate this explanation since initiation as measured by the synthesis of mRNA 5′-terminal di-, tri-, and tetranucleotides was differentially resistant to inhibition by AMT photoreaction. This finding is more consistent with an alternative possibility, that sites near the ends of the genome RNAs, perhaps adjacent to promoters, are highly susceptible to psoralen adduct formation. Thus, reiterative synthesis of mRNA 5′-terminal oligonucleotides, a process that may not require movement of the polymerase relative to the genome RNA, continues on modified templates. Elongation, on the other hand, is prevented by failure of the polymerase to pass sites of psoralen attachment, perhaps, as suggested for cytoplasmic poliovirus (21), because damaged genome RNA cannot move through the polymerase catalytic site.

A differential inhibitory effect on transcript elongation as compared to initiation as measured by mRNA 5′-terminal oligonucleotide synthesis was also observed with reovirus cores that were inactivated by dimethyl sulfate alkylation of the genome RNA. Again the diminished yield of RNA products consisted of full length rather than prematurely terminated molecules. Similarly, UV irradiation at λ_{260 nm} markedly diminished reovirus transcription without yielding incomplete transcripts (data not shown) (22). However, this was apparently not, as suggested (22), due to a block in initiation since oligonucleotide yields were relatively unaffected (G-C, G-C-U, and G-C-U-A = 146, 87, and 75% of control, respectively) by UV irradiation of cores for 2 min, conditions that reduced formation of full length mRNAs by 92% (data not shown). These findings suggest that individual polymerase molecules enter the elongation phase of transcription only if the template RNA is unmodified and, thus, functionally open along its entire length.

Initiation of reovirus transcription results in the synthesis of an excess of short oligonucleotides corresponding to the common 5′-terminal sequence of the multiple species of viral mRNA (14, 15, 29). This suggests that the reovirus polymerase (or a distinct primase activity) repeatedly transcribes pro-
motors sites as described recently for E. coli DNA-dependent RNA polymerase (5). The reovirus oligonucleotides are produced not only in incomplete reaction mixtures lacking 1 or 2 ribonucleoside triphosphates but also under conditions of mRNA synthesis. In the latter case only a fraction of the oligonucleotides are elongated. The capped oligonucleotides apparently are used preferentially in vitro for chain extension to form full length capped mRNAs. Overproduction of uncapped 5'-terminal oligonucleotides that are not incorporated into mRNAs *in vivo* until possibly late in the reovirus replicative cycle (24), may account for most of the single-stranded oligonucleotides present in mature reovirions (25).

Recent reports of transcription in a variety of other eukaryotic systems suggest that excess initiation may be a general characteristic of mRNA synthesis. The RNA polymerase in the double-stranded RNA virus of *Bombyx mori*, insect cytoplasmic polyhedrosis virus, produces a mixture of capped and uncapped di- and trinucleotides *in vitro* (6). As observed for human reovirus, the insect virus transcriptase products are made in high molar excess relative to mRNA, correspond mainly to the mRNA 5'-terminal sequence, and are differentially resistant to psoralen photoinactivation (6). The DNA-dependent RNA polymerase in vaccinia virus also synthesizes low molecular weight species of RNA under some conditions (26, 27). These RNAs are initiated from the multiple promoters in vaccinia DNA, contain 5'-terminal caps and are prematurely terminated and polyadenylated. Most of the vaccinia products are longer, but some have chain lengths of only about twenty nucleotides which suggests that the nonpoly(A), 5' portions of the short transcripts may be analogous to the oligonucleotides described here. Synthesis of vesicular stomatitis virus mRNAs by the virion-associated polymerase *in vitro* is also accompanied by the production of an excess of short oligonucleotides and at least three distinct small RNA species (chain lengths 28, 42, and 70 nucleotides) (28). The small RNAs correspond in sequence to the 5' termini of three of the five individual mRNAs. Their formation, which apparently occurs by attenuation of mRNA chain growth, was previously found to be resistant to psoralen photoreactivation under conditions that inactivated the synthesis of viral mRNAs (29). These findings are consistent with the presence of psoralen-susceptible site(s) located beyond the promoter(s) for mRNA synthesis in the vesicular stomatitis virus genome. Thus initiation of mRNA synthesis can occur in AMT-photoreacted vesicular stomatitis virus but entry into the elongation phase of transcription is blocked.

In addition to the excess initiations characteristic of *E. coli* RNA polymerase and virion-associated polymerases *in vitro*, RNA polymerase II products in the nuclei of uninfected HeLa and Chinese hamster ovary cells (30, 31) and adenovirus-infected HeLa cells (32, 33) include a high proportion of prematurely terminated, promoter-proximal transcripts. Gel analyses of a fraction of the in vitro products showed that they are in the size range of ~80–300 nucleotides. In these studies shorter oligonucleotides resulting from mRNA initiation events and which may also be normal products of polymerase II would not have been detected by the experimental protocols employed. It will be of interest to test whether RNA polymerase II not only prematurely terminates at distinct sites to form abortive transcripts of chain length ~100 or more, but also if an excess of oligonucleotides is synthesized by reiterated initiation at promoter sites.

**Note Added in Proof**—Purified reovirions (not treated with chymotrypsin) incubated in reaction mixtures containing Mn²⁺ and 4 rNTPs synthesized the same capped and uncapped 5'-terminal oligonucleotides but no mRNA.

**REFERENCES**

1. Furuichi, Y., Muthukrishnan, S., Tomasz, J., and Shatkin, A. J. (1976) *J. Biol. Chem.* 251, 5043-5053
2. Shatkin, A. J. (1976) *Cell* 9, 645-653
3. Darnell, J. E. (1979) *Prog. Nucleic Acid Res. Mol. Biol.* 22, 327-353
4. Donie-Keller, H., Maxam, A. M., and Gilbert, W. (1977) *Nucleic Acids Res.* 4, 2527-2536
5. Carpousis, A. J., and Grada, J. D. (1980) *Biochemistry* 19, 3245-3253
6. Furuichi, Y. (1981) *J. Biol. Chem.* 256, 483-493
7. Shatkin, A. J., and LaFliandra, A. J. (1972) *J. Virol.* 10, 698-706
8. Furuichi, Y., Morgan, M., Muthukrishnan, S., and Shatkin, A. J. (1975) *Proc. Natl. Acad. Sci. U. S. A.* 72, 382-386
9. Shintsi, H., Miwa, M., Sugimura, T., Shimotohno, K., and Miura, K. (1976) *FEBS Lett.* 65, 254-257
10. Nakashima, K., and Shatkin, A. J. (1978) *J. Biol. Chem.* 253, 8680-8682
11. Nakashima, K., LaFiandra, A. J., and Shatkin, A. J. (1979) *J. Biol. Chem.* 254, 8007-8014
12. Bishop, D. H. L. (1977) in *Comprehensive Virology* (Fraenkel-Conrat, H., and Wagner, R. R., eds.) Vol. 10, pp. 117-278, Plenum Press, New York
13. Bellamy, A. R., and Hole, L. V. (1979) *Virology* 40, 808-819
14. Kozak, M. (1977) *Nature* 269, 390-394
15. Zarbl, H., Hastings, K. E. M., and Millward, S. (1980) *Arch. Biochem. Biophys.* 202, 348-360
16. Isaacs, S. T., Shen, C. J., Hearst, J. E., and Rapeport, H. (1977) *Biochemistry* 16, 1058-1064
17. Bordlin, F. S., Marciani, F. R., Baccichetti, F., Dall'Acqua and Rodighiero, G. (1975) *Ital. J. Biochem. (Engl. Ed.)* 24, 258-267
18. Peattie, D. A., and Gilbert, W. (1980) *Proc. Natl. Acad. Sci. U. S. A.* 77, 4679-4682
19. Joklik, W. K. (1974) in *Comprehensive Virology* (Fraenkel-Conrat, H., and Wagner, R. R., eds.) Vol. 2, pp. 231-334, Plenum Press, New York
20. Silverstein, S. C., Christman, J. K., and Aris, G. (1976) *Annu. Rev. Biochem.* 45, 375-406
21. Yazaki, K., and Miura, K.-I. (1980) *Virology* 105, 467-479
22. Henderson, D. R., and Joklik, W. K. (1978) *Virology* 91, 389-406
23. Hastings, K. E. M., and Millward, S. (1978) *J. Virol.* 28, 490-498
24. Zarbl, H., Skup, D., and Millward, S. (1980) *J. Virol.* 49, 497-505
25. Stolfzus, C. M., and Banerjee, A. K. (1972) *Arch. Biochem. Biophys.* 152, 723-734
26. Paulettri, E., Lipinskas, B. R., and Panical, D. (1980) *J. Virol.* 33, 208-219
27. Gershowitz, A., and Moss, B. (1979) *J. Virol.* 31, 849-853
28. Testa, D., Chanda, P. K., and Banerjee, A. K. (1980) *Cell* 21, 267-275
29. Nakashima, K., Chanda, P. K., Deutsch, V., Banerjee, A. K., and Shatkin, A. J. (1979) *J. Virol.* 32, 838-844
30. Tam, L., and Kikuchi, T. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 76, 5750-5754
31. Salblitt-Georgiief, M., Harpold, M., Chen-Kiang, S., and Darnell, J. E., Jr. (1980) *Cell* 19, 69-76
32. Fraser, N. W., Sehgal, P. B., and Darnell, J. E., Jr. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 76, 2571-2575
33. Evans, R., Weber, J., Ziff, E., and Darnell, J. E., Jr. (1979) *Nature* 287, 367-370

R. E. Smith and Y. Furuichi, unpublished results.