JOURNAL OF VIROLOGY, July 1991, p. 3964–3967
0022-538X/91/073964-04$02.00/0

Vol. 65, No. 7

Photoaffinity Labeling of Rotavirus VP1 with 8-Azido-ATP: Identification of the Viral RNA Polymerase

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Received 20 December 1990/Accepted 15 April 1991

Rotavirus single-shelled particles have several enzymatic activities that are involved with the synthesis of capped mRNAs both in vivo and in vitro. Because single-shelled particles must be structurally intact to carry out transcription, it has proven to be difficult to identify the protein within such particles that possesses associated RNA polymerase activity. One approach for characterizing the function of the individual proteins within single-shelled particles is to use nucleotide analogs to specifically label those proteins, such as the viral RNA polymerase, that have affinity for nucleotides. In this study, 8-azido-ATP (azido-ATP), a photoreactable nucleotide analog, was used to identify the viral RNA polymerase on the basis of the ability of the analog to inhibit transcription activity associated with rotavirus particles on exposure to UV light. When single-shelled particles were treated with UV light in the presence of [α-^{32}P]azido-ATP, the structural protein VP1 became radiolabeled because of cross-linking of the nucleotide analog, and there was a corresponding decrease in the ability of the particles to synthesize mRNA. In parallel experiments in which single-shelled particles were not exposed to UV light, VP1 was not radiolabeled and the particles successfully used azido-ATP as a substrate for the synthesis of viral mRNAs. Taken together, these results are consistent only with the conclusion that VP1 is the rotavirus RNA-dependent RNA polymerase.

Rotaviruses, members of the family *Reoviridae*, are major causes of gastroenteritis in young children and animals (5). The genome of the rotaviruses consists of 11 unique segments of double-stranded RNA, each of which encodes a different protein (1, 14). Infectious rotavirus virions are made up of two icosahedral shells of protein. Within the inner shell is a core that consists of the genome, the major core protein VP2 (102 kDa), and the minor core proteins VP1 (125 kDa) and VP3 (98 kDa). VP6 (45 kDa) surrounds the core, forming the icosahedral inner shell of the virus. Particles that lack the outer shell of protein, single-shelled particles, have an associated RNA polymerase activity able to support the synthesis of viral mRNA in vitro (3). Synthesis of mRNAs by single-shelled particles is dependent on the presence of VP6, as removal of this protein produces core particles that are transcriptionally inactive (2, 21, 22). Reconstitution of cores with VP6 restores associated polymerase activity (2, 22). Transcripts made by single-shelled particles in vitro are identical to those made in vivo, containing 5′-terminal cap structures but lacking 3′-terminal polyadenylate (9, 25).

Efforts have been made to identify those viral proteins that have associated enzyme activities important for rotavirus genome replication and transcription (5, 18). Previous studies have shown that VP3 possesses associated guanylyltransferase activity (19), VP2 has affinity for both single-stranded and double-stranded RNA (12), and VP6, for unknown reasons, is an essential component of particles with transcriptase activity (2, 21, 22). The viral protein that functions as the RNA polymerase to synthesize viral mRNAs and double-stranded RNAs has yet to be demonstrated. However, because VP1 has sequence homology with RNA polymerases of other RNA viruses and is in low copy number in the virion, VP1 has been proposed to function as the rotavirus RNA polymerase (4–7). Consistent with this proposal is the finding that VP1, in addition to the viral proteins VP2 and VP3 and the nonstructural proteins NS35 and NS34, is a component of the subviral particles that have associated replicase activity (7, 8).

The intact structure of the rotavirus single-shelled particle is necessary to transcribe the viral genome in vitro as well as in vivo (7, 23). Attempts to correlate enzymatic activities with the protein components of the single-shelled particle by using purified proteins have been unsuccessful, yielding results similar to those obtained with other viruses of the same family (10). In characterizing the enzyme activities associated with viral proteins, two approaches have been used to label proteins with affinity for nucleotides. In the first approach, the protein can be identified by using a radiolabeled form of its natural substrate. Such is the case for rotavirus guanylyltransferase, which in the presence of [α-^{32}P]GTP becomes radiolabeled as a result of a covalent complex formed between the enzyme and GMP. In the second approach, a radiolabeled nucleotide analog can be induced to covalently cross-link to the nucleotide-binding site of a protein, resulting in not only the labeling of the protein but also the loss of associated enzyme activity (19). These approaches are useful for characterizing rotavirus proteins involved with mRNA synthesis, since they do not require the disruption of transcriptionally active particles. The nucleotide analog 8-azido-ATP (azido-ATP) has proven to be useful for the identification of proteins, such as DNA-dependent RNA polymerase, that use nucleotides as substrates, since the analog is capable of binding covalently to nucleotide-binding sites in proteins upon exposure to UV light (11, 17, 20). The photochemically generated nitrene of the analog reacts with amino acid residues that are proximal to the binding site, forming a complex between the nucleotide and the protein and allowing the identification of the cata-

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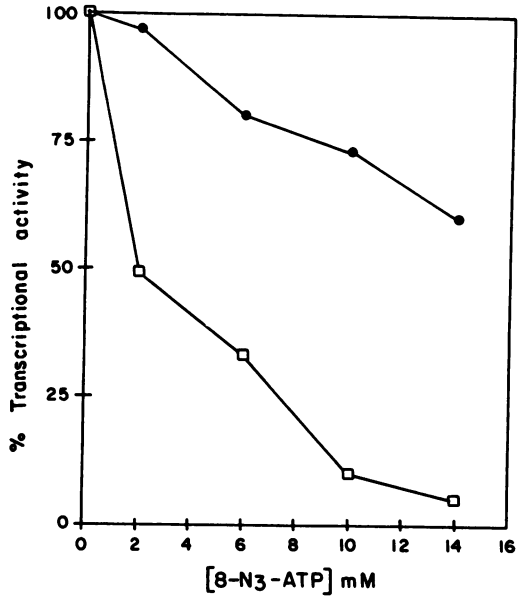

FIG. 1. Effect of azido-ATP (8-N_3-ATP) on rotavirus transcription. Heat-activated rotavirus (1 min at 55°C) was incubated with azido-ATP on ice for 5 min and then exposed (□) or not exposed (●) to UV light for 1.5 min at a distance of 4 cm. Afterward, the virus was assayed for transcription activity by incubation for 30 min at 45°C, and the acid-precipitable radioactivity was measured. The results are expressed as the percentage of the [^{3}H]UMP incorporated into acid-insoluble material. One hundred percent of transcription activity corresponds to 300 pmol of incorporation. Similar levels of incorporation were obtained in assays done in the absence of azido-ATP with or without UV irradiation.

lytic protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (13, 26). One of the advantages of working with this kind of analog is that it does not modify the native structure of the protein, even if the associated enzymatic activity of the protein becomes inhibited (20).

The purposes of this study were to determine whether the rotavirus protein that has RNA polymerase activity could be identified by specific binding of azido-ATP to the enzyme and to test whether binding of the analog correlated with an inhibitory effect on viral RNA synthesis.

Effect of azido-ATP on transcription. The effect of azido-ATP on rotavirus in vitro transcription was studied by using a purified human rotavirus isolate (23, 24). Because of the sensitivity of the nucleotide analog to light, all experiments were performed in darkness, except as noted (20). Heat-activated virus was incubated on ice for 5 min in reaction mixtures containing various amounts of azido-ATP and then irradiated with UV light (254 nm) for 1.5 min at a distance of 4 cm. Reaction mixtures prepared similarly but not exposed to UV light served as controls. After UV irradiation, the virus was assayed for associated transcriptase activity by adding 15 μl of a solution containing 160 mM Tris-HCl (pH 8.5); 10 mM $MgCl_2$; 80 mM NaCl; 2 mM (each) ATP, CTP, and GTP; 0.24 mM [^{3}H]UTP (50 cpm/pmol); and 0.1 mM *S*-adenosylmethionine. After incubation for 30 min at 45°C, the reaction mixtures were assayed for acid-precipitable ^{3}H-labeled product. Control experiments showed that in the absence of azido-ATP, exposure of activated rotavirus to UV light had no effect on the level of virus-associated RNA polymerase activity (Fig. 1). UV exposure of reaction mixtures containing increasing concentrations of azido-ATP decreased viral transcription in a dose-dependent manner (Fig. 1). A concentration of 2 mM inhibited rotavirus transcription by 55%, whereas a concentration of 14 mM inhibited transcription by 95%. The requirement for a high concentration of azido-ATP to produce 95% inhibition was probably due to either a high rate of degradation of the analog under the assay conditions or, more likely, a difficulty of the analog to reach the target site within the virus particle (12, 26). In reaction mixtures not exposed to UV light, inhibition of virus transcription occurred at high concentrations of the analog (>6 mM), although to a significantly lower degree than that seen for parallel reaction mixtures exposed to UV light. Similar inhibition also occurs in transcription reaction mixtures that contain high concentrations of ATP (data not shown). Together, these results demonstrate that exposure to UV light in the presence of the analog azido-ATP inhibited in vitro transcription by rotavirus.

Photoaffinity labeling of rotavirus proteins with [α-^{32}P] azido-ATP. To determine whether the inhibitory effect on transcription produced by azido-ATP as a result of exposure to UV light correlated with the covalent cross-linking of the analog to a viral protein, experiments using [α-^{32}P]azido-ATP were performed. SA11 rotavirus grown in MA-104 cell culture or human rotavirus isolated from stool samples was purified by centrifugation of CsCl gradients (21). Single-shelled and core particles were prepared from purified virus as previously described (22). Heat-activated double-shelled virus, single-shelled virus, and core particles were incubated with 1 μCi of [α-^{32}P]azido-ATP (89.3 Ci/ml; ICN) for 5 min at 4°C and then irradiated with UV light for 1.5 min. The photoaffinity-labeling reactions were terminated by the addition of 0.1 mM EDTA. The reaction mixtures were boiled in Laemmli sample buffer and subjected to electrophoresis on a 12% polyacrylamide gel (13, 16).Rotavirus proteins that became radiolabeled as a result of UV irradiation were detected by exposure of the gel to X-ray film for 24 h at −20°C. As shown by the autoradiogram in Fig. 2, exposure of heat-activated double-shelled human rotavirus (0.1 μg) to UV light resulted in the radiolabeling of only a single species of protein (lane 2). In the absence of UV irradiation, no radiolabeled proteins were detected (lane 1). The same protein became radiolabeled upon exposure of SA11 and human single-shelled virus as well as viral cores to UV light (lanes 3 to 6). The labeled protein migrated with an apparent molecular mass of 125 kDa when compared with molecular mass standards. In comparison with ^{35}S-labeled SA11 virus, the radiolabeled protein comigrated precisely with VP1. Therefore, the results show that under the assay conditions employed in this study, VP1 is the sole viral protein to which azido-ATP can cross-link upon exposure of rotavirus to UV light.

Ability of [α-^{32}P]azido-ATP to serve as a substrate during in vitro transcription. The results described above show that the azido analog, upon exposure to UV light, inhibited polymerase activity associated with rotavirus particles and cross-linked covalently to VP1. As a test of the ability of the RNA polymerase to use azido-ATP as a nucleotide substrate, heat-activated human rotavirus was incubated on ice for 5 min with various concentrations of both virus and [α-^{32}P]azido-ATP. Next, some samples were irradiated with UV light for 1.5 min. Both the UV-treated and untreated samples were then assayed for RNA polymerase activity for 30 min at 45°C. Afterward, sample buffer containing 99% formamide, 0.25 M EDTA (pH 8.0), and 0.01% xylene cyanol was added to the reaction mixtures. The ^{32}P-labeled

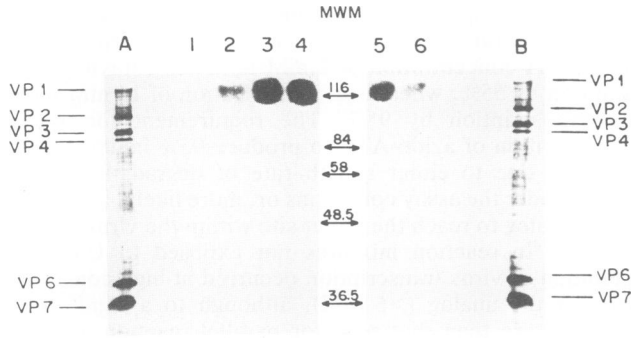

FIG. 2. Photolabeling of rotavirus proteins with [α-^{32}P]azido-ATP. Rotavirus particles were incubated with [α-^{32}P]azido-ATP for 5 min on ice and then, in some cases, exposed to UV light. Double-shelled human rotavirus (0.1 μg) was incubated with the azido analog and not irradiated (lane 1) or irradiated (lane 2) with UV light. Rotavirus single-shelled particles and cores were prepared as described elsewhere (23). Human single-shelled particles (0.2 μg; lane 3) and cores (0.2 μg; lane 4) were incubated with the azido analog and exposed to UV light for 1.5 min at a distance of 4 cm. SA11 single-shelled particles (0.05 μg; lane 5) and cores (0.04 μg; lane 6) were incubated with analog and exposed to UV light as described above. Samples were analyzed by SDS-PAGE and processed for autoradiography. Lanes A and B indicate the positions of ^{35}S-labeled viral proteins as determined by coelectrophoresis of SA11 virions. The positions of molecular weight standards (in thousands) are indicated (MWM).

RNA products of the reactions were analyzed by electrophoresis on 5% polyacrylamide gels containing 8 M urea and processed for autoradiography. As shown in Fig. 3, reactions containing rotavirus and [α-^{32}P]azido-ATP which had not been exposed to UV light synthesized radiolabeled transcripts (lanes 1 to 4). Hence, the RNA polymerase within rotavirus particles used azido-ATP as a substrate for the synthesis of viral mRNA. Virus exposed to UV light in the presence of [α-^{32}P]azido-ATP before the assay for polymerase activity was unable to synthesize viral mRNAs (lanes 5 to 8). Virus exposed to UV light in the presence of [α-^{32}P]azido-ATP for suboptimal lengths of time (15 to 60 s) before the assay for RNA polymerase activity synthesized radiolabeled transcripts at levels inversely proportional to the time of irradiation with UV light (Fig. 3, lanes 9 to 11).

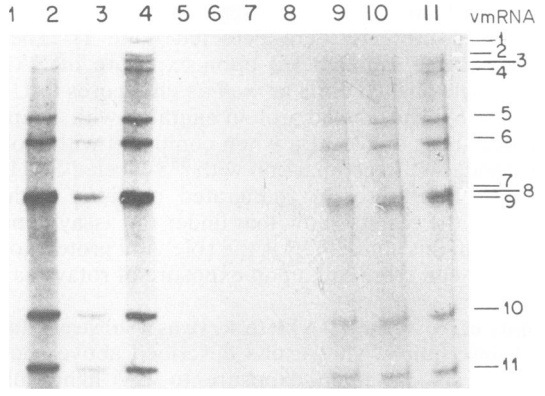

FIG. 3. [α-^{32}P]azido-ATP as a substrate for viral polymerase. Either 0.1 μg (lanes 1, 2, 5, and 6) or 0.2 μg (lanes 3, 4, 7, and 8) of heat-activated double-shelled human rotavirus was incubated on ice for 5 min with 0.1 μCi (lanes 1, 3, 5, and 7) or 0.2 μCi (lanes 2, 4, 6, and 8) of [α-^{32}P]azido-ATP and then not irradiated (lanes 1 to 4) or irradiated (lanes 5 to 8) with UV light. Double-shelled rotavirus (0.1 μg) was incubated with 0.2 μCi of [α-^{32}P]azido-ATP and exposed to UV light for 60 s (lane 9), 45 s (lane 10), or 15 s (lane 11). Afterward, the virus was assayed for transcription activity. Products of reactions were analyzed for the presence of [^{32}P]azido-AMP-labeled mRNAs by electrophoresis on a 5% polyacrylamide gel containing 8 M urea and by autoradiography. vmRNA, viral mRNA.

We have shown that azido-ATP is used by the rotavirus RNA polymerase during transcription as a nucleotide substrate. Therefore, the azido analog must have the ability to interact with the nucleotide-binding domain of the RNA polymerase. The fact that exposure to UV light in the presence of azido-ATP inhibited the ability of activated rotavirus to synthesize mRNAs indicates that azido-ATP becomes cross-linked to the nucleotide-binding domain in the presence of light, thus rendering the RNA polymerase nonfunctional. The only protein that became radiolabeled as a result of exposure of double- and single-shelled virus and core particles to UV light in the presence of [α-^{32}P]azido-ATP was VP1. We conclude from these data that VP1 is responsible for binding of nucleotides during transcription for the purpose of providing the substrate during RNA polymerization and thus is in fact the viral RNA polymerase. This conclusion is consistent with sequence analysis, which shows that VP1 has four regions of amino acid homology that are conserved among RNA-dependent RNA polymerases of numerous viruses (15). Our results are inconsistent with the possibility that VP3 functions as the viral RNA polymerase, despite sequence analysis which indicates that VP3 may have some homology with various other viral RNA polymerases. Because rotavirus transcription is associated only with structurally intact single-shelled particles, it seems apparent that other viral proteins such as VP3, the guanylyltransferase, must interact either with the RNA template or with VP1 in such a way as to provide a function that is essential for polymerase activity. In contrast to rotavirus transcription, the template for genome replication is single stranded and the RNA polymerase that synthesized double-stranded RNA (replicase) does not require VP6 for activity. These properties raise the possibility that the protein that functions as the RNA polymerase during transcription is different from that which functions during genome replication. However, because VP1 is a component of particles with replicase activity, it seems reasonable to predict that VP1 functions as the viral replicase. Studies are under way to determine whether azido-ATP can be cross-linked to the VP1 protein by exposure of enzymatically active replicase particles to UV light.

We thank P. Ortiz for his technical assistance.

This work was supported by grants from FONDECYT (1017-89) and SAREC (Sweden) to E.S. and from the National Institutes of Health (AI21478 and AI00990) to J.P.

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