Removal of double-stranded contaminants from RNA transcripts: synthesis of adenovirus VA RNA_I from a T7 vector

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ABSTRACT

Bacteriophage RNA polymerases are widely used to synthesize defined RNAs on a large scale *in vitro*. Unfortunately, the RNA product contains a small proportion of contaminating RNAs, including complementary species, which can lead to errors of interpretation. We cloned the gene encoding Ad2 VA RNA_I into a vector containing a T7 RNA polymerase promoter in order to generate large quantities of VA RNA for the study of its interaction with the dsRNA-dependent protein kinase DAI. Exact copies of VA RNA_I were synthesized efficiently, but were contaminated with small amounts of dsRNA which activated DAI and confounded interpretation of kinase assays. We therefore developed a method to remove the dsRNA contaminants, allowing VA RNA_I and mutants to be tested for their ability to activate or inhibit DAI. This method appears to be generally applicable.

INTRODUCTION

VA RNA was discovered as an abundant RNA present in KB cells infected with adenovirus type 2 (Ad2; ref. 1). Subsequently it was realized that there are two such RNAs, VA RNA_I and VA RNA_{II}, both about 160 nucleotides in length (2). The RNAs accumulate to high levels (about 10^8 and 10^7 copies per cell, respectively) in the cytoplasm of infected cells (3), and their sequences suggest that they can adopt stable secondary structures (4,5). They are encoded by closely spaced genes located at approximately map unit 30 on the adenoviral genome (2,6). Transcription of the genes by RNA polymerase III begins at early times of infection and accelerates during the late phase (3,7,8).

The role of the VA RNAs in translational control was revealed through the study of mutants which carry disruptions of the transcriptional signals located within the genes (9). Protein synthesis was reduced about 10-fold in cells infected with an adenovirus mutant that cannot make VA RNA_I, but proceeded normally in cells infected with a mutant virus unable to transcribe VA RNA_{II}. This indicates that VA RNA_I is the more important as well as the more abundant species. In cells infected with a doubly mutant virus, where both VA RNAs were missing, protein synthesis was reduced 50-fold, suggesting that VA RNA_{II} can partially substitute for VA RNA_I (10). Further studies demonstrated that VA RNA antagonizes the activation of a cellular protein kinase known as DAI, the double stranded RNA (dsRNA) dependent inhibitor of protein synthesis (11,12). This enzyme is present in many cell types at a low level, and its synthesis is induced by interferon (13,14). When activated by dsRNA in a process involving autophosphorylation, DAI inhibits protein synthesis by phosphorylating the initiation factor eIF-2, thereby trapping a second initiation factor (GEF or eIF-2B). VA RNA_I prevents the dsRNA-mediated activation of DAI, preserving protein synthesis in the infected cell and functioning as a viral defense mechanism to oppose the effects of interferon (15,16).

VA RNA_I binds to DAI both *in vivo* and *in vitro* and can block the binding of the dsRNA activator (17–19). Regions of the VA RNA molecule involved in binding DAI *in vitro* and required for preventing its activation *in vivo* have been defined through the use of mutants (20–22). Further progress in studying the structure and function of the molecule depends on a convenient method for obtaining substantial quantities of VA RNA or of its mutant derivatives. Kinase inhibition assays and attempts at crystallization, in particular, require large amounts of RNA. To this end, we constructed a vector that contains a promoter for the bacteriophage T7 RNA polymerase, allowing synthesis of correctly initiated and terminated VA RNA_I. This polymerase displays a high degree of promoter specificity and is easily purified from bacteria which overexpress the polymerase gene (23,24). Optimized transcription reactions produced copious quantities of authentic VA RNA_I. However, the product also contained small amounts of contaminating dsRNA. Because DAI is activated by very low concentrations of dsRNA, these contaminants obscured the results of kinase inhibition assays. We therefore devised a procedure to purify VA RNA_I away from the interfering contaminants, so that it can be tested for its ability to inhibit the activation of DAI. This procedure appears to be broadly applicable to RNA synthesized *in vitro*.

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MATERIALS AND METHODS

Vector construction

Plasmid pT7VA/Ad2I, abbreviated here to pT7VA, is a derivative of the cloning vector pUC119 containing the promoter for T7 RNA polymerase (derived from pET-7; ref. 25) fused upstream of the gene for Ad2 VA RNA_I (from pMHVA; ref. 20). Run-off transcription using T7 RNA polymerase produces exact copies of VA RNA_I beginning at the major (G) start of transcription and terminating with three uridylate residues at the 3' end (Figure 1). The plasmid contains the ϕ 10 promoter of phage T7 (nt −37 to +2, Bgl II-Stu I fragment of pET-7) inserted at the Bam HI site of the vector via a Bam/Bgl II fusion. The StuI half-site was fused to the VA RNA_I gene at nucleotide +3 after cleavage of a synthetically constructed Fsp I site introduced at the 5' end of the Ad2 VA RNA_I gene. Thus, the 5' junction is a*ggGCA*, where the Stu I and Fsp I half-sites are lower and upper case, respectively, and the transcribed portion is underlined. The 3' end of the VA RNA_I gene was modified from TTTTGG to TTTAAA using oligonucleotide-directed mutagenesis (26), creating a Dra I restriction endonuclease site. The downstream junction was formed by fusing a Sal I site introduced 70 bases downstream of the VA RNA_I gene to the Sal I site in the pUC119 polylinker.

Transcription reactions

T7 RNA polymerase was purified as described (24). Plasmid pT7VA DNA was banded twice in CsCl gradients, treated with 20 μg/ml RNase A for 30 min. at 37°C in TE (10 mM Tris−HCl, pH 8.0, 1 mM EDTA), deproteinized, then digested with Dra I. Transcription reactions contained 37.5 μg/ml T7 RNA polymerase, 50 μg/ml DNA, 40 mM Tris-HCl, pH 7.8, 14 mM $MgCl_2$, 2mM spermidine, 5 mM dithiothreitol (DTT), 4 mM each ribonucleoside triphosphate, and 1 unit/μl RNasin (Promega). A trace amount (50 μCi/ml) of α-^{32}P-GTP was added to follow the VA RNA through the purification scheme. Reactions were incubated at 40°C for 90 min., terminated by the addition of EDTA to 20 mM, then extracted once with phenol and once with chloroform. Transcription conditions were based on those previously reported (27) and were optimized for VA RNA_I yield by varying the temperature and time of incubation and the concentrations of the reaction components listed above. Substantial improvement in yield resulted from raising the nucleotide and $MgCl_2$ concentrations relative to standard conditions. Other components tested, including Triton X-100, PEG-8000, creatine phosphate plus creatinine kinase, Hepes-KOH and BSA, did not affect the RNA yield significantly.

Purification of VA RNA_I

VA RNA was recovered by ethanol precipitation, denatured in formamide loading solution, and fractionated in an 8% polyacrylamide/ 7M urea sequencing gel for 2.5 hours at 1.7 KV as previously described (20). The major band was excised and VA RNA was eluted by the crush and soak technique. The RNA was phenol extracted and precipitated with ethanol, then resuspended in glycerol loading solution (non-denaturing) and fractionated in an 8% polyacrylamide sequencing-type gel, cast without urea, for 6 hours at 500 volts. The RNA was recovered from the gel as before, dissolved in 35% ethanol/STE and loaded onto a cellulose CF-11 column (28). VA RNA was eluted as previously described (29) except that 22% ethanol/STE was employed instead of 15% ethanol/STE. The fractions containing the peak of radioactivity were pooled and the RNA was precipitated with ethanol and dissolved in water. The nucleic acid concentration was determined by spectrophotometry. Overall recovery of VA RNA_I after the three purification steps was 10−15% (i.e., 200−300 μg/100 μg plasmid DNA).

Kinase assay

DAI was purified to the hydroxylapatite stage (19). Kinase assays were performed using 1 μl of DAI fraction (134 μg/ml), 20 mM KCl, 5mM $MgCl_2$, 15 mM HEPES-KOH, pH 7.4, 1 mM DTT, 10 μM PMSF, 0.1 mM ATP, 250 μCi/ml γ-^{32}PATP (3000 Ci/mmole, Amersham), 1 μg/ml of aprotinin, leupeptin, and pepstatin, in a final volume of 20 μl. Reactions were incubated at 30°C for 20 min and terminated by the addition of an equal volume of 2× SDS-gel sample buffer. Samples were heated to 100°C for 2 minutes and fractionated in 12.5% SDS-polyacrylamide gels (30). Gels were fixed, dried and subjected to autoradiography. When necessary, the bands containing autophosphorylated DAI were excised and subjected to scintillation counting.

Synthesis of synthetic dsRNA

Double stranded RNA was prepared by hybridizing complementary strands transcribed *in vitro* from the plasmid pGEM.GC, essentially as published previously (19). This plasmid contains a 354 bp 'GC clamp' fragment (Xba I to Hind III) from pMHVA (20) inserted between the equivalent sites of pGEM1 (Promega). RNA generated by T7 RNA polymerase transcription

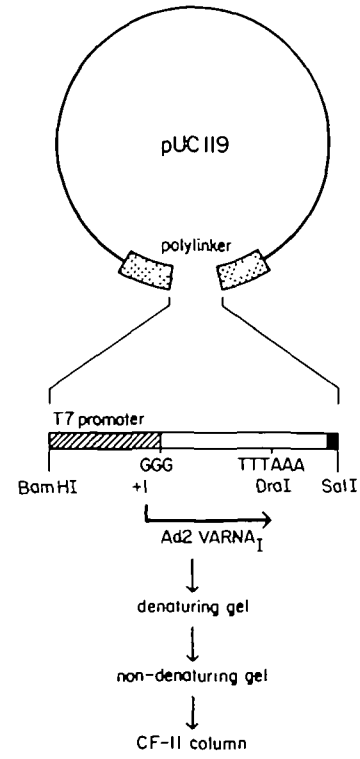

Figure 1. Plasmid construction and VA RNA purification scheme. Plasmid pT7VA contains the T7 RNA polymerase promoter (hatched) and the structural gene for adenovirus 2 VA RNA_I (open box) cloned into the polylinker of the vector pUC 119. An additional 21 nucleotide linker sequence (filled box) separates the Ad2 Bal I site (nucleotide 10810) from the Sal I site in the vector polylinker. A Dra I site allows transcription to runoff at the 3'-end of the VA RNA structural gene. The purification scheme developed here is outlined below.

of pGEM.GC DNA cleaved with Nae I was annealed to RNA generated by SP6 RNA polymerase transcription of pGEM.GC cleaved with Sph I. After digestion with RNase A (10 μg/ml), dsRNA 170 bp in length was purified by electrophoresis through an 8% polyacrylamide gel.

Ribonuclease digestion

RNA samples were digested with ribonuclease T_1 (RNAse T_1, Calbiochem) in TE buffer using an enzyme to substrate ratio of 1:10, or with ribonuclease III (RNase III, purified through the phosphocellulose stage; ref. 31) in a buffer containing 20mM Tris-HCl, pH 7.6, 100mM NH_4Cl, and 10mM Mg acetate (32) using 1.5 units of enzyme for 5 μg unpurified VA RNA or 10 ng reovirus dsRNA. Digestion was carried out for 30 min at 37°C. RNA was recovered by phenol extraction, chloroform extraction, and ethanol precipitation in the presence of 20 μg/ml glycogen as carrier. Recovery was monitored by Cerenkov counting.

RESULTS

Synthesis of VA RNA_I

To produce large amounts of Ad2 VA RNA_I *in vitro*, we constructed the plasmid pT7VA which abuts the VA RNA coding sequences with the T7 RNA polymerase promoter (Fig. 1). VA RNA_I extracted from adenovirus-infected cells exhibits heterogeneity at both its 5′ (33−35) and 3′ ends (36). The major initiation site is at a G residue which lies three nucleotides downstream from an A residue that serves as a minor start site. Because the mutant virus Ad5 dl309 is fully viable even though it fails to make A-start VA RNA, the predominant G-start form is sufficient for viral growth (37). VA RNA_I carries 2−6 uridylate residues at its 3′ terminus, with three in the most abundant form (36). Therefore, pT7VA was constructed such that T7 RNA polymerase initiates at the major (G) transcriptional start and terminates with three uridylate residues by running off at a Dra I site. Under optimal transcription conditions, reactions produced about 20 μg of VA RNA_I from 1 μg of linearized plasmid, a molar yield of 750. This represents a one thousand fold increase over the yield obtained with RNA polymerase III transcription extracts from 293 cells (data not shown). The VA RNA_I was indistinguishable from that isolated from infected cells by RNA fingerprinting (38) and nuclease sensitivity analysis (20).

Activity of VA RNA synthesized *in vitro*

In initial experiments, VA RNA_I was purified from the transcription reaction by fractionation through a denaturing polyacrylamide gel followed by a cellulose column designed to separate single-stranded from double-stranded RNA (28). This chromatographic procedure probably also removes impurities derived from the gel. The resultant RNA was assayed for its ability to inhibit activation of DAI. As shown in Fig. 2A, high concentrations of this RNA (20 and 50 μg/ml) blocked activation of DAI by reovirus dsRNA (Fig. 2A, lanes 2 and 3). Lower concentrations blocked to a lesser extent (Fig. 2A, lanes 4 and

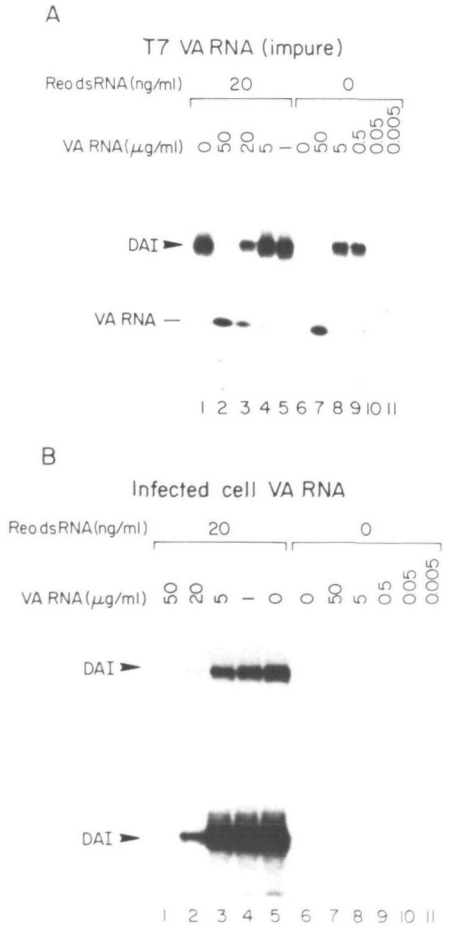

Figure 2. Activation and inhibition of DAI by VA RNA. Kinase assays contained (A) VA RNA_I produced *in vitro* by T7 RNA polymerase transcription and purified through a denaturing gel and CF11 column, or (B) VA RNA_I isolated from Ad2-infected HeLa cells (19). The quantities of RNA added to each reaction and the positions of VA RNA and autophosphorylated DAI are indicated. A 7-fold longer exposure of panel B is shown below.

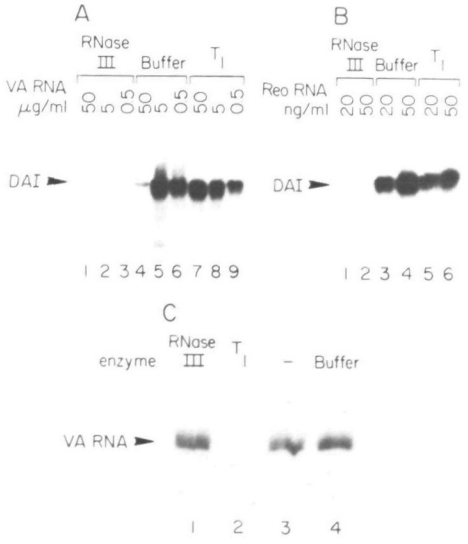

Figure 3. *Sensitivity of VA RNA and reovirus dsRNA to RNase T_1 and RNase III.* (A) VA RNA_I, purified as in Fig. 2 from the T7 transcription reaction, was incubated with buffer or enzyme, deproteinized, then added to kinase assays at the concentrations indicated. (B) Reovirus dsRNA was incubated with buffer or enzyme, deproteinized, then added to kinase assays at the concentrations indicated. Panels A and B are sections of the same autoradiogram. (C) Radiolabeled VA RNA was incubated with RNase III (lane 1), RNase T_1 (lane 2), sample buffer (lane 4), or not incubated (lane 3), and fractionated in a denaturing gel. The position of radiolabeled VA RNA_I is indicated.

5). In agreement with earlier reports (11,12), similar results were obtained with VA RNA_I isolated from Ad2-infected HeLa cells (Fig. 2B, lanes 1 and 2).

We also examined the ability of the two VA RNA preparations to activate DAI in the absence of reovirus dsRNA. Over a wide range of concentrations, the RNA isolated from infected cells activated DAI only very slightly. This weak activation was visible on an overexposed film at concentrations between 50 and 500 ng/ml VA RNA (Fig. 2B, lower panel, lanes 9 and 10). By contrast, VA RNA_I synthesized *in vitro* by T7 RNA polymerase activated DAI strongly at 0.5 and 5 μg/ml (Fig. 2A, lanes 8 and 9) although higher concentrations blocked the activation (Fig. 2A, lane 7). These results show that the VA RNA made *in vitro* is functional as an inhibitor of DAI activation and suggest that it is contaminated with an activator that is not removed by electrophoresis through a single gel and CF11 chromatography.

To test this conclusion and determine the nature of the activator, we incubated the VA RNA_I preparation with the enzymes RNase T_1 or RNase III, specific for single-stranded and double-stranded RNA, respectively. RNase T_1 cleaves RNA at unpaired guanosine residues (38), while RNase III digests perfectly duplexed RNA of ≥20 base pairs (and certain stem-loop structures present in some procaryotic RNA processing sites [39,40]). The specificity of each enzyme was authenticated by incubation with reovirus dsRNA and VA RNA_I. Radiolabeled VA RNA_I was degraded by RNase T_1 but not by RNase III (Figure 3C). Conversely, reovirus dsRNA lost its ability to activate DAI upon incubation with RNase III but not with RNase T_1 (Figure 3B), indicating digestion of reovirus dsRNA by RNase III but not RNase T_1. We then applied the same test to the VA RNA_I preparation used in Fig. 2A. Like reovirus RNA, the activation of DAI by VA RNA_I was sensitive to digestion with RNase III (Figure 3A, compare lanes 1−3) but not RNase T_1 (Fig. 3A, lanes 7−9). In fact, activation of DAI at high concentrations of RNA was increased by RNase T_1 digestion, probably because of the removal of VA RNA_I that had been masking the activity of the contaminant (Figure 3A, lanes 4 and 7). These results indicate that the activator which is present in the VA RNA_I preparation is probably dsRNA. Judging from the ability of the contaminant to activate DAI, we estimate that it represents approximately 0.1−1% of the total mass of RNA (compare Fig. 3A, lane 7 and Fig. 3B, lane 4).

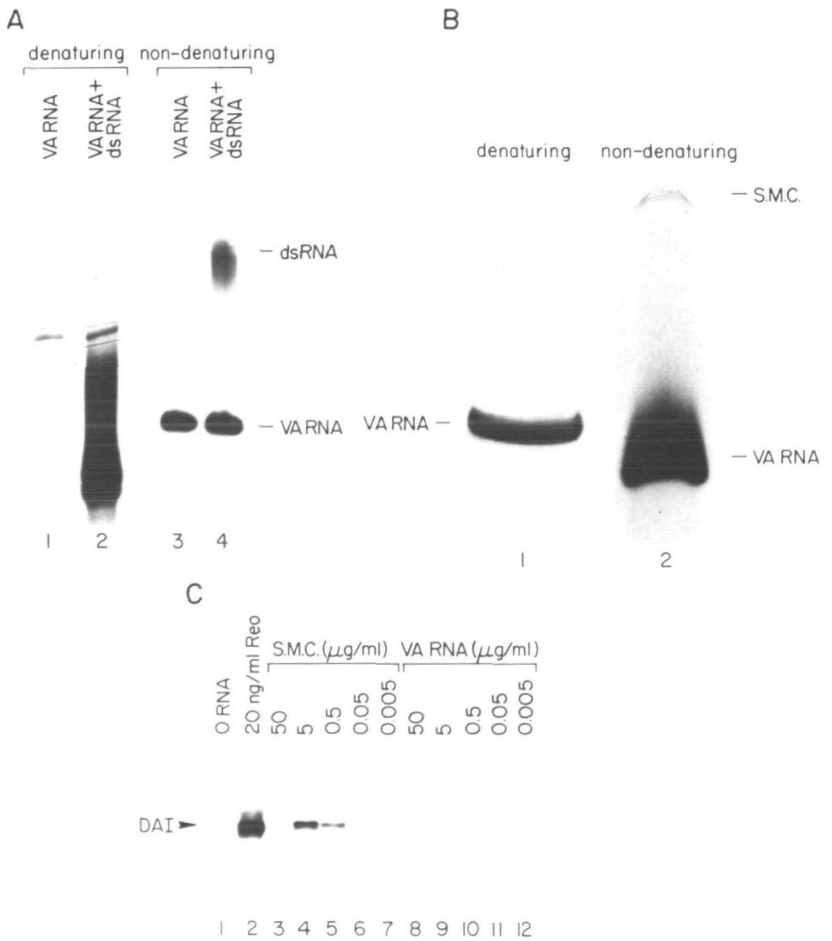

Figure 4. Rationale for purification procedure. (A) Radiolabeled VA RNA_I alone (lane 1), or mixed with labeled dsRNA (lane 2), was fractionated in an 8% polyacrylamide/7M urea sequencing gel. Sections of the gel containing VA RNA (outlined) were excised. The RNAs recovered from lanes 1 and 2 were then fractionated in lanes 3 and 4, respectively, of a non-denaturing polyacrylamide gel. The positions of the VA RNA and dsRNA are indicated. (B) Preparative denaturing sequencing gel containing the RNA from transcription of 100 μg of Dra I linearized pT7VA was fractionated in a denaturing gel (lane 1). The VA RNA was eluted, recovered and further purified in a non-denaturing gel (lane 2). The positions of VA RNA and a slowly migrating contaminant (S.M.C.) are indicated. (C) VA RNA and the S.M.C. were purified as in panel B from a functional mutant of VA RNA (sub 118−119, Mellits, Pe'ery and Mathews, unpublished results) and were tested for their ability to activate DAI.

Purification of VA RNA_I

We wanted to develop a procedure to remove the contaminant which did not rely on digestion with the enzyme RNase III as a purification measure. Although the contaminant copurified with VA RNA_I in a denaturing polyacrylamide gel, in view of its structure we thought it unlikely that the two RNAs would also co-migrate in a non-denaturing gel. To test this idea, VA RNA was loaded onto a denaturing gel either alone or together with partially degraded synthetic dsRNA, intended to simulate the dsRNA contaminant (Fig. 4A, lanes 1 and 2). After electrophoresis, the RNA migrating in the same area as VA RNA_I in each lane was excised and eluted (as outlined in Figure 4, lanes 1 and 2). The RNAs recovered were then loaded into separate lanes of a non-denaturing polyacrylamide gel; in this gel, the band corresponding to synthetic dsRNA migrated considerably more slowly than VA RNA_I (Figure 4A, lanes 3 and 4).

We therefore fractionated VA RNA_I synthesized from pT7VA in a denaturing polyacrylamide gel and then in a non-denaturing gel (Fig. 4B). When the non-denaturing gel was overexposed, a minor band migrating more slowly than VA RNA_I was detected. This material was present in approximately the same quantity as the proposed contaminant, and is referred to as the slow migrating contaminant (S.M.C.). To discover whether this material could be responsible for the activation of DAI observed with partially purified preparations of VA RNA_I, we eluted the S.M.C. from a non-denaturing gel and performed kinase assays. This experiment was conducted with a VA RNA_I mutant, sub 118−119, which gave increased amounts of the S.M.C. band but behaves like wild-type VA RNA in kinase inhibition assays (Mellits, Pe'ery and Mathews, unpublished data). As shown in Figure 4C, the S.M.C. activated DAI (Fig. 4C, lanes 3−8), particularly at 5 and 0.5 μg/ml, whereas the corresponding VA RNA band was not able to efficiently activate the kinase (Fig. 4C, lanes 8−12). These data demonstrate that the vast majority of the activating contaminant was removed from the mutant VA RNA preparation by electrophoresis through a non-denaturing gel, and that at least some of the activator can be purified as the S.M.C. band.

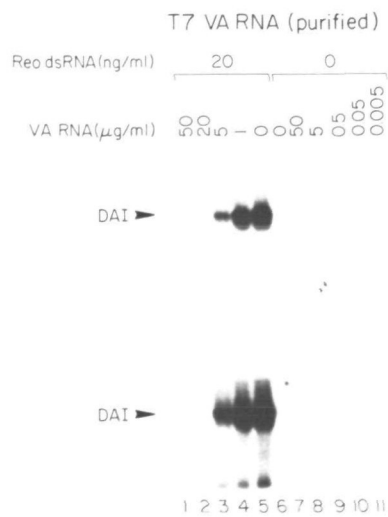

Figure 5. Activities of purified VA RNA_I synthesized *in vitro* after purification. Wild-type VA RNA_I synthesized by T7 RNA polymerase was purified through denaturing and non-denaturing gels and a CF-11 column. The resultant RNA was tested for its ability to activate (lanes 6−11) or prevent activation (lanes 1−5) of DAI in kinase assays. The lower panel is a 4-fold longer exposure of the upper panel.

Based on these findings, we employed sequential electrophoresis through denaturing and non-denaturing polyacrylamide gels to purify wild-type VA RNA_I. As an additional measure we subjected the eluted RNA to fractionation on cellulose columns. The resultant RNA was tested for its ability to inhibit and to activate DAI (Fig. 5). A comparison between Figures 5 and 2B indicates that the purified synthetic VA RNA_I behaves similarly to VA RNA_I purified from adenovirus 2 infected cells, in both DAI activation and inhibition assays. Most importantly, it exhibits significantly less activating activity than synthetic VA RNA_I purified only through a denaturing gel and cellulose column (cf. Fig. 2A, lanes 7−11).

DISCUSSION

We have constructed a vector, pT7VA, which utilizes a phage T7 promoter to overproduce adenovirus VA RNA_I. The vector also allows site-specific mutations to be introduced by oligonucleotide-directed mutagenesis. Unfortunately, dsRNA is produced during the synthesis of VA RNA by run-off transcription with T7 RNA polymerase. This contaminant interferes with assays of the DAI inhibitory function of the RNA product by introducing additional quantities of an activator. The effects can be particularly confusing because the activation of DAI is extremely sensitive to low levels (10−50 ng/ml) of dsRNA and high concentrations of dsRNA (1−10 μg/ml) inhibit activation of the enzyme (41−43). Therefore, for use in kinase assays, it is crucial that RNAs produced from such vectors be purified away from dsRNA contaminants. To accomplish this, we developed a general procedure involving electrophoresis through sequential denaturing and non-denaturing polyacrylamide gels followed by a simple chromatographic step (see Figure 1).

At least some of the dsRNA that is generated during the transcription reaction co-purifies with VA RNA_I in a denaturing sequencing gel and can be visualized as a more slowly migrating band in a subsequent non-denaturing sequencing gel. The source of this slowly migrating contaminant is unknown. Some preparations of T7 RNA polymerase efficiently replicate a short RNA (X RNA) in an apparently template independent fashion during transcription of DNA templates (44). The replication of X RNA involves synthesis of complementary RNAs, each of which has a hairpin structure. These strands then anneal yielding a duplexed RNA. Although VA RNA_I has a considerable degree of secondary structure (20), it did not serve as template to generate an 'X' type VA RNA when incubated with our T7 RNA polymerase (data not shown). Because the yield of the slowly migrating contaminant seemed to depend on the template used, it is more likely to be transcribed from DNA. Low levels of spurious DNA-dependent transcripts complementary to runoff transcripts are produced by both SP6 and T7 RNA polymerases (45,46). The synthesis of such transcripts is increased when short templates with 3′ overhanging ends are transcribed, but is also observed at low levels (<1%) from DNA templates with 5′ overhanging ends or blunt ends (as used here). In our preparations, we estimate the dsRNA contamination level to be approximately 0.1−1%. It is thought that these anti-sense transcripts are formed when some RNA polymerase molecules do not 'run off' the linearized DNA but instead swing around and continue transcription on the opposite DNA strand (46). Alternatively, the antisense RNA may be generated from cryptic

promoters located on complementary strands of the plasmid sequences.

Given the sensitivity of DAI to low concentrations of dsRNA, contamination at a level of less than 1% could produce enough dsRNA to activate DAI. Removal of the contaminant by the procedure we have developed relies largely on the differential mobility of the VA RNA and dsRNA species in non-denaturing and denaturing conditions (Fig. 4; ref. 47). It is likely that the aberrant electrophoretic mobility of VA RNA_I also contributes to the separation: VA RNA_I migrates with single-stranded RNA molecules of ~220 nt. in a denaturing gel and of ~135 nt. in a non-denaturing gel (Pe'ery, Mellits and Mathews, in preparation). Whatever the mechanism, it is likely that the procedure described here can be used to purify a wide variety of small transcripts, particularly those produced by *in vitro* transcription with bacterial RNA polymerase, for use in kinase inhibition and other assays. A case in point is the recent demonstration (48) that purification of HIV-1 TAR RNA by the procedure described here successfully removes dsRNA contaminants that give spurious DAI activation. Furthermore, this procedure may be useful for removing complementary strand contaminants from single-stranded RNA hybridization probes.

After purification, the VA RNA_I synthesized by T7 RNA polymerase behaves like the authentic molecule purified from infected cells in that neither of them activates DAI to a significant extent (11,12). We estimate that, at its peak, the level of activation observed with VA RNA is less than 1% of that obtained with 20 ng/ml reovirus dsRNA. Short perfect duplexes, of a length comparable with the duplex regions in VA RNA, also activate DAI poorly if at all (49–51; L. Manche and M.B. Mathews, unpublished observations). These results are set against those of Galabru et al. (18) who found that VA RNA_I made *in vivo* activated DAI to a significant degree (approximately 10% as well as reovirus dsRNA; M. Katze, personal communication). It is not clear whether the discrepancy is due to different assay conditions, dsRNA contamination, or other factors. Our findings are consistent with the view that adenovirus-2 VA RNA_I is a specialized effector designed to bind to the dsRNA binding site of DAI in such a way as to prevent rather than promote activation of the enzyme (22).

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