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Regulation of a Double-Stranded RNA Modification Activity in Human Cells

LOUISA M. MORRISSEY AND KARLA KIRKEGAARD*

Howard Hughes Medical Institute and Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80309

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A double-stranded RNA (dsRNA)-specific modification activity from *Xenopus* oocytes and human cells (dsRNA modifier) converts adenosine residues present in dsRNA to inosines. The function of the dsRNA modifier is unknown, although it has been suggested that it may be part of the cellular antiviral response. We investigated the relationship between the activity of the dsRNA modifier, viral infection, and the antiviral response in human cells induced by poly(rI)-poly(rC) [poly(I · C)] treatment. We found, unexpectedly, that treatment of HeLa cells with poly(I · C) or other dsRNA molecules resulted in the dramatic inhibition of the dsRNA modifier. Mixing experiments, reconstruction experiments, and pretreatment of extracts with RNases indicated that inhibition of the dsRNA modifier did not result from the continued presence of a soluble inhibitor (such as dsRNA) in the in vitro modification reactions. Treatment of cells with cyclohexamide or dactinomycin simultaneously with the poly(I · C) demonstrated that in vivo inhibition of the dsRNA modifier did not require new transcription or translation. The dsRNA modification activity was also substantially inhibited in cells infected with poliovirus and was slightly inhibited in cells infected with adenovirus. The inhibition of the dsRNA modifier during the antiviral state is thus not consistent with an antiviral function, and instead suggests another cellular function for dsRNA modification.

A double-stranded RNA (dsRNA) modification activity (dsRNA modifier) from *Xenopus* eggs (4) and mammalian cells (41), originally termed an RNA-unwinding activity (3, 32, 40), converts adenosines to inosines in RNA duplexes (4, 41). The adenosine-to-inosine conversion both destabilizes the RNA double helix and alters the informational content of the RNA (1). The level of dsRNA modification activity, its intracellular location, or both vary during both *Xenopus* development (3, 32) and the cell cycle of mouse fibroblast 3T3 cells (40, 41).

The cellular function of the dsRNA modifier remains unknown. dsRNA modification has been suggested to function in natural antisense regulation of gene expression in *Xenopus* (17), pathogenicity in defective measles virus (5, 8, 9, 44), and posttranscriptional modification of hepatitis delta virus RNA (26). It has also been suggested that the dsRNA modifier constitutes part of the cellular antiviral response (21, 41), since modification of viral RNAs when they are in a double-stranded conformation might destroy their structure, informational content, or both. We tested a possible antiviral role of the dsRNA modifier by examining its relationship to the known cellular antiviral response.

Mammalian cells constitutively express low levels of proteins with known antiviral functions, including a dsRNA-dependent protein kinase and the dsRNA-dependent 2′,5′-oligo(A) synthetase (24, 30). Type I interferons, which are secreted proteins that protect homologous cells against viral infections, are not normally present in uninfected cells. Synthesis of interferon is induced in many cell types by either viral infection or treatment with synthetic or natural dsRNA (24, 30). Other genes are also induced by these treatments (11, 39, 42, 47), and interferons in turn induce the transcription of many genes with antiviral functions, including the dsRNA-dependent protein kinase and 2′,5′-oligo(A) synthetase (12, 33–35, 45). The actions of interferons in the antiviral response are known to be dependent upon new transcription. In the absence of ongoing transcription, mammalian cells still display many responses to inducers of the antiviral state, such as poly(I)-poly(C) [poly(I · C)], a synthetic dsRNA. These responses include activation of the dsRNA-dependent protein kinase and 2′,5′-oligo(A) synthetase present constitutively in the cell (24, 30), stimulation of the transcription factor NF-κB (23), and possibly activation of other constitutively synthesized cellular proteins as well (13). Although many of the transcriptional responses to poly(I · C) do not occur readily in HeLa cells, the known posttranslational responses to poly(I · C) do occur (10).

We investigated the relationship of the dsRNA modifier in HeLa cells with some of the known components of the human antiviral response. We found that treatment of human HeLa cells with poly(I · C) to induce the antiviral state resulted in the inhibition of the dsRNA modification activity as assayed subsequently in vitro. This observation suggests that, far from being a component of the antiviral response in mammalian cells, the dsRNA modifier has a normal cellular function that is inhibited during the antiviral response. Consistent with this idea, the dsRNA modifier was also found to be inhibited during infection with poliovirus, a single-stranded RNA virus whose infection can lead to the accumulation of intracellular dsRNA (6, 7, 29). The dsRNA modifier was significantly less inhibited during infection with adenovirus, a virus whose virus-associated (VA) RNA products are known to subvert the cellular antiviral response (36).

MATERIALS AND METHODS

Cells, viruses, and extracts. HeLa cells were grown as described previously (18) and were treated with 600 μg of DEAE-dextran (Sigma) per ml with or without 100 μg of poly(I · C) (Pharmacia) per ml for 2 h at 37°C in serum-free

* Corresponding author.

TABLE 1. Percentage of adenosine-to-inosine modification of a radiolabeled dsRNA substrate in extracts from HeLa cells subjected to a variety of conditions in the presence or absence of poly(I-C)

Treatment (duration)[a]	% of adenosine-to-inosine modification of a dsRNA substrate	
	Without poly(I · C)	With poly(I · C)
None	9.4	1.2
	9.5	0.6
	7.1	0.6
Dactinomycin	9.4	1.1
Cyclohexamide (2 h)	8.9	2.1
Cyclohexamide (4 h)	7.6	NT[b]
β-Interferon	7.0	0.7

[a] The inhibition of RNA and protein synthesis, respectively, by dactinomycin and cyclohexamide under these conditions was tested in parallel experiments (Materials and Methods).
[b] NT, not tested.

DME (Dulbecco modified Eagle medium). Additional treatments were as follows: 500 U of β-interferon (Lee Biomolecular) per ml for 6 h prior to the addition of poly(I · C); 1 μg of dactinomycin (Sigma) per ml for 1 h prior to the addition of poly(I · C); 50 μg of cycloheximide per ml concurrently with poly(I · C) treatment; 100 μg of poly(rA)-poly(rC) (Pharmacia) per ml in the presence of 600 μg of DEAE-dextran per ml in serum-free DME for 2 h at 37°C; and 100 μg of sonicated salmon sperm DNA (Sigma) per ml with 600 μg of DEAE-dextran per ml for 2 h at 37°C.

The efficacies of these concentrations of dactinomycin and cylohexamide on the inhibition of RNA and protein synthesis, respectively, were tested in separate experiments. These tests were performed in parallel to experiments similar to those reported in Table 1, and similar inhibitions of the dsRNA modifier were observed. HeLa cells treated with 1 μg of dactinomycin per ml as described above were found to incorporate 11% of the amount of [^{3}H]uridine (Dupont, NEN Research Products) incorporated by untreated cells in 2 h at 37°C; poly(I · C) did not affect the inhibition of RNA synthesis by dactinomycin. HeLa cells treated with 50 μg of cyclohexamide per ml as described above (except that the DME contained no methionine) incorporated 5% as much [^{35}S]methionine (Dupont, NEN) as did untreated cells in 2 h at 37°C. Poly(I · C) did not affect the inhibition of protein synthesis by cyclohexamide.

To quantify the amount of poly(I · C) remaining in the Manley extracts, poly(I · C) was 5′ end-labeled with ^{32}P to a specific activity of 21,000 cpm/μg of poly(I · C). Cells were treated with this ^{32}P-labeled poly(I · C) as described for unlabeled poly(I · C), and Manley extracts were prepared. A 10-μl sample of extract was found to contain 148 cpm, or 6.1 ng, of residual poly(I · C); inhibition of dsRNA modification activity was observed in these extracts.

Infections with Mahoney type 1 poliovirus were performed at MOI of 40 as described previously (18). Infections with phenotypically wild-type dl309 adenovirus serotype 5 (obtained from J. Schaak, University of Colorado Health Sciences Center) were performed at a multiplicity of infection (MOI) of 5 as described previously (16). Manley whole-cell extracts were prepared from batches of 10^8 HeLa cells as described previously (27).

dsRNAs. Poly(I · C), a synthetic dsRNA, was purchased from Pharmacia. dsRNA, approximately 4,600 bp in length, from viruslike particles of *Saccharomyces cerevesiae* was isolated from the L-A-containing strain TF229 as described by Icho and Wickner (15). The viruslike particle dsRNA was further purified by phenol extraction and $LiCl_2$ precipitation (15).

The dsRNA substrate for the in vitro modification assay was prepared by annealing complementary single-stranded RNA transcripts made by SP6 and T7 RNA polymerases (Boehringer), respectively, from plasmid pG158 or pGB. pG158 contains an approximately 158-bp *Sca*I-*Eco*RI DNA fragment cloned into pGem3 (Promega Biotec). The 158-bp insert was derived from pPolio (18) and includes nucleotides 7332 to 7440 of Mahoney type 1 poliovirus cDNA (20, 31), 20 nucleotides each of poly(A) and poly(C), as well as *Cla*I and *Eco*RI linker DNA sequences. pGB contains a 286-bp *Xba*I-*Bam*HI fragment derived from pPolio cloned into pGem4 (Promega Biotec). The 286-bp fragment contains nucleotides 4600 to 4886 of Mahoney type 1 poliovirus cDNA (20, 31). Transcription reactions were performed as described previously (46); 275 pmol of [α-^{32}P]ATP (800 Ci/mmol; Dupont, NEN) was included in the reaction with SP6 polymerase. The transcripts were hybridized in 80% formamide and 300 mM NaCl for 16 h at 55°C. The RNA hybrids were subsequently digested with RNase A and RNase T1 (46) and purified following electrophoresis on a 6% polyacrylamide gel.

In vitro dsRNA modification assays. From 30 to 300 pmol of the duplex RNA was incubated at 30°C in 20-μl reactions containing various cell extracts and 10 mM MgCl, 20 mM phosphocreatine, 10 mM KCl, 10 mM ATP, and 10 mM Tris, pH 7.4 (final concentrations). The concentration of dsRNA substrate was varied between experiments because of differences in the specific activities of individual RNA preparations; however, the 1.5 to 15 μM range of substrate concentrations used was well below saturation conditions for the RNA modification activity in the extracts (data not shown). Reactions were terminated with proteinase K, the mixtures were extracted with phenol, and the RNAs were collected by ethanol precipitation and digested with P1 nuclease as described previously (4). Samples were spotted on polyethyleneimine (PEI) cellulose plates (EM Science) and developed in either solvent A [saturated $(NH_4)_2SO_4$–0.1 M sodium acetate, pH 6.0–isopropanol, 79:19:2 (vol/vol/vol)] or solvent B (1.0 M acetic acid). The two different solvent systems yielded similar results; data from experiments using solvent A are presented here. The percentage of dsRNA modification was determined by scintillation counting of the excised 5′-AMP and 5′-IMP spots from thin-layer chromatographic analysis.

RESULTS

Treatment of HeLa cells with dsRNA inhibits the dsRNA modifier. Whole-cell extracts (27) made from HeLa cells or from HeLa cells pretreated in vivo with poly(I · C) were incubated in vitro with a 158-bp dsRNA substrate which was internally radiolabeled at adenosine residues. The dsRNA substrate was then digested to nucleoside 5′-monophosphates and analyzed by thin-layer chromatography (4). After 15 min of incubation with HeLa cell extracts, conversion of adenosine to inosine was readily observed. However, no such modification was seen after incubation with extracts from cells pretreated with poly(I · C) (Fig. 1A). Quantitation of these data revealed that, even after 2 h of incubation,

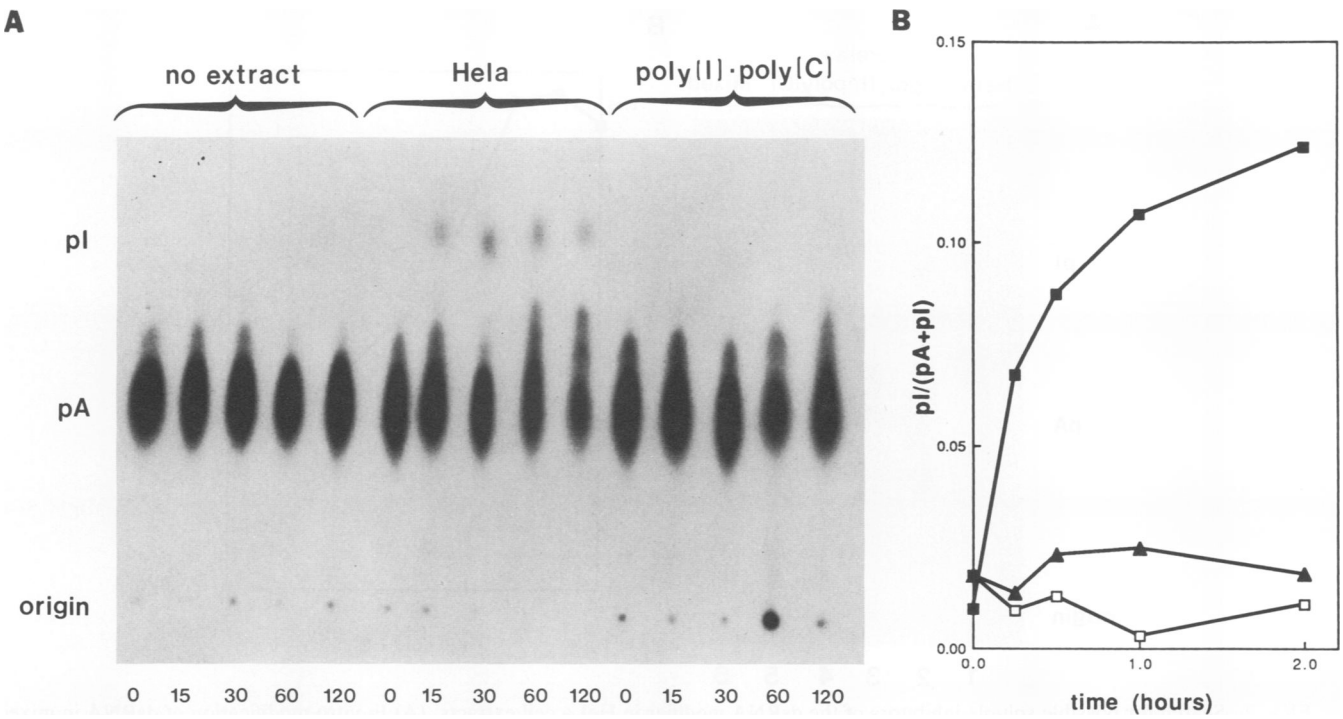

FIG. 1. Thin-layer chromatogram showing the inhibition of dsRNA modification by poly(I · C). (A) A ^{32}P-labeled dsRNA substrate made from pG158 was incubated with extracts prepared from HeLa cells treated with DEAE-dextran alone or in combination with poly(I · C). Samples with no extract contained Manley dialysis buffer (27). Following incubation for the time (in minutes) indicated below the lanes, dsRNA was digested to nucleoside 5′ monophosphates and analyzed by thin-layer chromatography (4). The positions of the 5′-AMP and 5′-IMP markers are indicated by pA and pI, respectively. (B) Kinetics of in vitro modification of the dsRNA substrate in whole-cell extracts. Symbols: □, no extract; ■, extract from cells treated with only DEAE-dextran; ▲, extract from cells treated with poly(I · C) and DEAE-dextran.

dsRNA modification could not be detected above background in extracts from HeLa cells treated in vivo with poly(I · C) (Fig. 1B).

To test whether the inhibition of the dsRNA modifier by poly(I · C) was, like other cellular responses to poly(I · C), specific for dsRNA (24, 30), we tested the effects of other polynucleotides on dsRNA modification. The dsRNA modifier was also inhibited in HeLa cells treated with dsRNA derived from yeast viruslike L-A particles (15; data not shown). However, treatment of HeLa cells with similar concentrations of single-stranded RNA [poly(rA · rC)] or dsDNA (salmon sperm) had no effect on dsRNA modification as subsequently assayed in vitro. In experiments in which extracts from HeLa cells treated only with DEAE-dextran showed 10.8% conversion of adenosine to inosine, extracts from cells treated with poly(A · C) and salmon sperm DNA showed 11.4 and 8.4% adenosine-to-inosine conversion, respectively (see Materials and Methods). Thus, the dsRNA modifier is inhibited when HeLa cells are treated with dsRNA, and not when they are treated with other polynucleotides we have tested.

Inhibition of the dsRNA modifier by poly(I · C) occurred in vivo. One explanation for the inhibition of the dsRNA modifier by treatment of cells with poly(I · C) was that a soluble component [poly(I · C), for example] remaining in the extract inhibited the dsRNA modifier activity during its assay in vitro. Equal volumes of extracts from HeLa cells and from HeLa cells treated with poly(I · C) were mixed and assayed for modification of the radiolabeled dsRNA substrate. dsRNA modification in HeLa cell extracts was not inhibited in the mixed extracts (Fig. 2). Preincubation of the active and inactive extracts together for 1 to 2 h prior to the addition of the radiolabeled dsRNA substrate still revealed no inhibition of the active extract (data not shown), thus showing that even after extended incubation, the constituents of the inactive extract did not inhibit the dsRNA modification activity in the active extract. In fact, to inhibit dsRNA modification in active extracts to background levels in vitro, it was necessary to add 1 μg of poly(I · C) to the reaction mixtures, to a final concentration of 50 μg/ml (Fig. 2B). When ^{32}P-labeled poly(I · C) was used in the cell treatments, only 6 ng (0.3 μg/ml) of residual ^{32}P-labeled poly(I · C) remained in 10 μl of extract, more than 100-fold less than the amount necessary to cause the levels of inhibition observed (see Materials and Methods). In addition, inactive extracts were preincubated with cobra venom ribonuclease (25) to degrade any preexisting dsRNA, and the ribonuclease was inactivated before the assay was performed. Such treatment did not restore dsRNA modification to inactive extracts. We concluded that there was no soluble inhibitor present in the inactive extracts and that the inhibition of dsRNA modification in those extracts was due to the response of the HeLa cells during in vivo treatment with dsRNA.

Poly(I · C) inhibits the dsRNA modifier in HeLa cells on the posttranslational level in vivo. Treatment of mammalian cells with poly(I · C) or any dsRNA has a variety of effects, such as the transcriptional induction of many genes, including that for interferon (11, 39, 42, 46, 47). Interferon in turn mediates the transcriptional activation of several other genes (12,

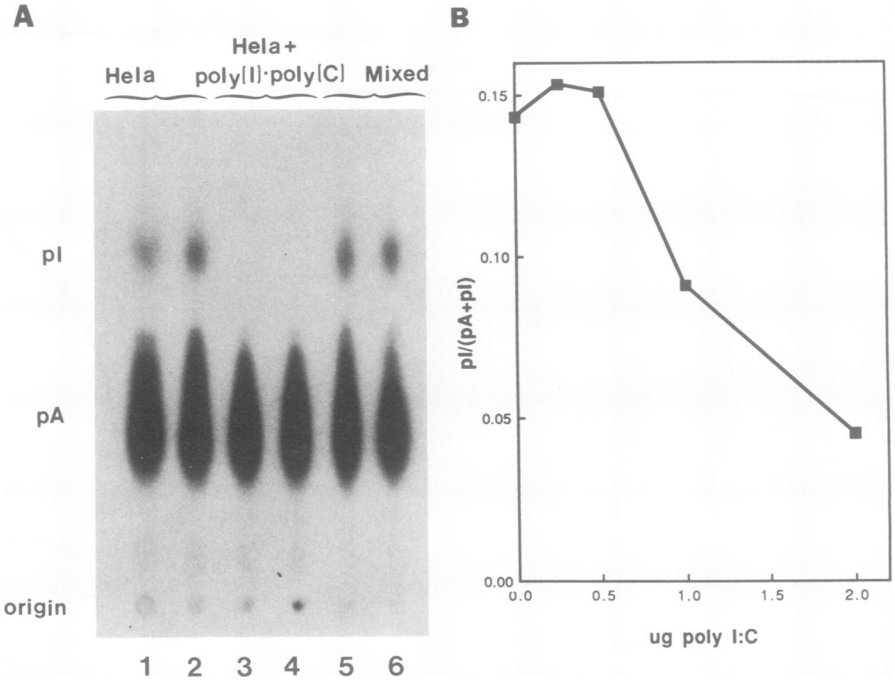

FIG. 2. Search for possible soluble inhibitors of the dsRNA modifier in HeLa cell extracts. (A) In vitro modification of dsRNA in mixed cellular extracts. Duplicate modification reactions are shown for extracts from DEAE-dextran-treated HeLa cells (lanes 1 and 2), HeLa cells treated with DEAE-dextran and poly(I · C) (lanes 3 and 4), and a mixture of equal volumes of these two extracts (lanes 5 and 6). Modification reactions were incubated for 30 min and were performed as described for Fig. 1, except that in reactions with mixed extracts, 10 μl of each extract or of Manley dialysis buffer was added in a final reaction volume of 25 μl. (B) In vitro inhibition of dsRNA modification by direct addition of poly(I · C) to HeLa cell extracts. Poly(I · C) in the amounts shown was added to extracts from HeLa cells treated with DEAE-dextran. Subsequent 1-h incubations with radiolabeled dsRNA substrate from pG158 were followed by determination of the percentage of conversion of adenosine to inosine by thin-layer chromatography.

33–35, 45). Cellular responses to dsRNA that are not dependent on new transcription or translation include the activation of 2′,5′-oligo(A) synthetase, of dsRNA-dependent protein kinase (DAI) (24, 30), and of NF-κB, a transcription factor (22).

To understand the relationship between the cellular response to poly(I · C) and its in vivo inhibition of the dsRNA modifier, we investigated the role of new macromolecular synthesis in this inhibition. We found that treatment of cells with dactinomycin, cyclohexamide, or β-interferon had little effect on either the dsRNA modification activity or its inhibition by poly(I · C) (Table 1). Therefore, the activity itself is stable, and its inhibition by dsRNA does not require new transcription, new translation, or the induction of interferon.

Poliovirus infection inhibits the dsRNA modifier. To test the idea that the inhibition of this activity is a bona fide cellular response to intracellular RNAs, we studied the effects of infection with an RNA virus on the dsRNA modifier. Figure 3 shows the effect of infection with poliovirus, a positive-strand RNA virus, on the dsRNA modification activity. By 3 to 5 h postinfection with Mahoney type 1 poliovirus, substantial inhibition of the dsRNA modifier had occurred.

The effects of poliovirus infection on HeLa cells include the inhibition of cellular translation (38). The inhibition of the dsRNA modifier by poliovirus infection was not due to this inhibition of cellular translation, however, because treatment of cells with cyclohexamide for the same 5-h period did not inhibit the activity (Table 1).

It is likely that poliovirus infection inhibits the dsRNA modifier by the in vivo production of double-stranded poliovirus RNA. Others have shown that some posttranslational components of the cellular response to poly(I · C) are stimulated during poliovirus infection, suggesting that sufficient dsRNA to activate these responses must be present. For

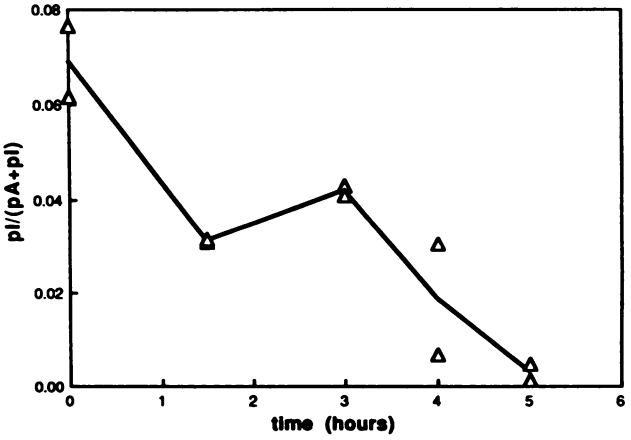

FIG. 3. Effects of poliovirus infection on the dsRNA modifier. HeLa cells were infected for the times shown with Mahoney type 1 poliovirus at an MOI of 40. Extracts were made by the method of Manley et al. (27). Duplicate reactions containing radiolabeled dsRNA substrate from pGB were assayed for conversion of adenosine to inosine by quantitative thin-layer chromatography.

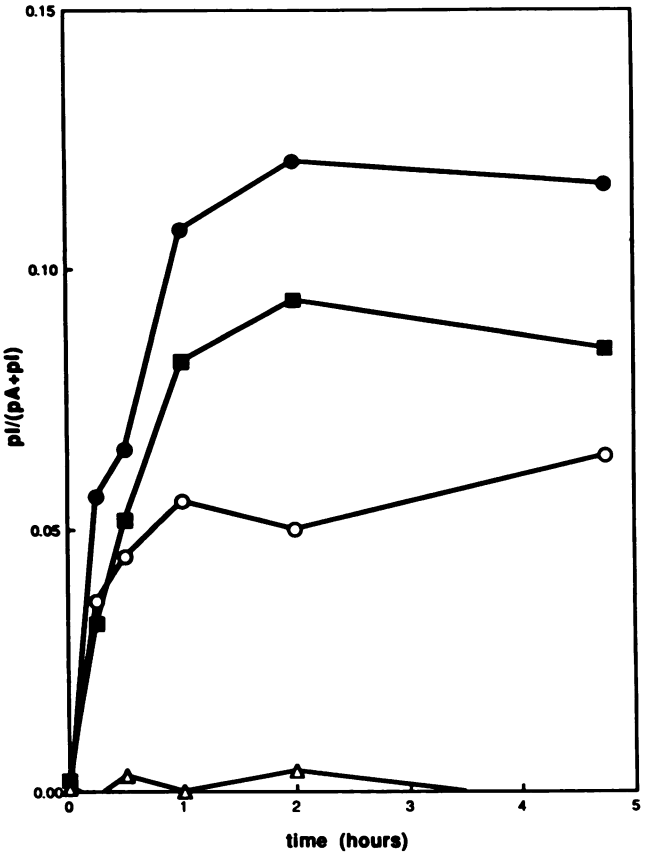

FIG. 4. In vitro time course of the dsRNA modifier activity in extracts from uninfected HeLa cells and from HeLa cells infected with poliovirus or adenovirus. HeLa cells were infected with either Mahoney type 1 poliovirus for 5 h at an MOI of 40 or with phenotypically wild-type dl309 adenovirus for 0 or 15 h at an MOI of 5. Incubation of the dsRNA substrate from pGB with the extracts was for the times indicated. ■, extract from HeLa cells treated with DEAE-dextran; ●, extract from mock-infected HeLa cells infected with adenovirus for 0 h; ○, extract from HeLa cells infected with adenovirus for 15 h at 37°C; △, extract from HeLa cells infected with poliovirus for 5 h at 37°C.

example, in HeLa cells infected with a poliovirus mutant defective in the inhibition of cellular translation, viral translation was found to be inhibited eventually by the cell (6). Furthermore, the activation and destabilization of the DAI has been observed during poliovirus infection (7, 29).

The finding that poliovirus infection inhibits the dsRNA modifier is consistent with the hypothesis that this inhibition occurs cellularly and is mediated by dsRNA. This observation does not, however, shed light on which of the known posttranslational responses to dsRNA, if any, mediates the inhibition of dsRNA modification.

Adenovirus infection does not substantially inhibit the dsRNA modification activity. We tested the in vivo effect of infection by adenovirus, a DNA virus, on the dsRNA modifier. Figure 4 shows the in vitro time course of dsRNA modification activity in extracts from untreated HeLa cells, HeLa cells infected with poliovirus for 5 h, and HeLa cells infected with adenovirus for 0 and 15 h. Little inhibition of dsRNA modification was seen in cells infected for 15 h with wild-type adenovirus; viral RNA synthesis should be maximal at this time (14). Analysis of infections performed in parallel revealed nearly 100% cytopathology by 40 h postinfection (data not shown). That adenoviral infection does not inhibit the dsRNA modifier, whereas poliovirus infection does, could provide an important clue to the mechanism of the inhibition of the dsRNA modifier by dsRNA in the cell. During infection of susceptible host cells with wild-type adenovirus, small RNAs termed VA1 and VA2 are synthesized at high levels and block the activation of DAI by dsRNA (19, 28, 36, 37). If the dsRNA modifier were inhibited by activation of DAI by poly(I · C), then the infection with adenovirus would prevent the inhibition of the dsRNA modifier, because the VA RNAs would prevent the activation of DAI. This and other hypotheses concerning the mechanism of inhibition of dsRNA modification activity by poly(I · C) treatment of cells are currently under investigation.

DISCUSSION

The existence in a variety of organisms of a dsRNA-specific activity that converts adenosines to inosines has prompted a great deal of speculation concerning its possible function. Since these adenosine-to-inosine conversions alter both the structure of a duplex RNA region and the coding capacity of that RNA, it is not clear which, if either, of these two functions is the more important (2). As a helix destabilizer, the dsRNA modifier could serve to modulate or reverse RNA-RNA interactions in the cell (2, 21) or to target dsRNA for degradation (17). In its role in the alteration of structure-specific information, the dsRNA modifier could serve as an RNA editor (43).

The idea that dsRNA modification might provide an antiviral function in mammalian cells derives primarily from work with defective measles virus genomes. For example, cDNAs made from RNA found in a measles inclusion body in an encephalitis patient showed that 132 out of 266 uridine residues had been converted to cytosine; this biased hypermutation could very easily have resulted from adenosine-to-inosine conversions in vivo on the opposite strand (8, 9). Furthermore, in the small RNA genome of hepatitis δ, a population of antigenomic RNA molecules was found to contain a specific base change that could have resulted from adenosine-to-inosine modification, even when the original RNA population did not bear the mutation (26). In this case, the mutation altered a stop codon, allowing the translation of a longer hepatitis δ protein (26). Knowledge of the difference in function, if any, of the two forms of the viral protein is necessary to know whether the modification event was hindering the virus or helping viral replication with this editing function.

We have shown that the dsRNA modifier in HeLa cells is inhibited following treatment with dsRNA. Treatment of HeLa cells with dsRNA is known to induce many components of the antiviral response typical of mammalian cells (10, 24, 30). That the cell inhibits the dsRNA modifier during its antiviral state leads to certain limitations on the possible cellular functions of dsRNA modification. It seems improbable that the cell would inhibit the dsRNA modifier during its antiviral response if the activity were indeed an important component of that response. Instead, our data suggest that dsRNA modification must have an important function in uninfected cells, and that its primary purpose is probably not antiviral.

As mentioned previously, it has been proposed (21, 41) that one of the constitutive functions of dsRNA modification might be to destabilize inter- and intramolecular dsRNA

structures. We suggest that the destabilization of RNA duplexes by dsRNA modification might afford protection against misfolding of cellular RNAs which, if they contained dsRNA regions of sufficient length, would inappropriately activate the cell's antiviral response. Thus, dsRNA modification activity might function in RNA structure repair by disrupting intramolecular duplexes in the cell.

Such an activity would become counterproductive, however, during viral infection, when dsRNA should serve as a signal to the cell that viral replication is occurring. It is possible that only high intracellular concentrations of dsRNA inhibit the dsRNA modifier, a possibility that we are currently investigating. The purpose of inhibiting the dsRNA modifier, for example during viral infection, would therefore be to prevent the dsRNA modification activity from serving its normal RNA structure repair function and to allow the cellular antiviral response to proceed.

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