Antiviral Research, 7 (1987) 317–327
Elsevier

AVR 00244

Priming of influenza mRNA transcription is inhibited in CHO cells treated with the methylation inhibitor, Neplanocin A

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(Received 18 December 1986; accepted 25 March 1987)

Summary

Chinese hamster ovary cells were pretreated with Neplanocin A, a potent inhibitor of RNA methylation. Analysis of polyadenylated RNA from treated cells by high-pressure liquid chromatography revealed marked decreases of 2′-*O*-methylation within mRNA cap structures and of internal N^6-methyladenosine residues. In these Neplanocin A-treated cells, influenza viral mRNA accumulation was virtually abolished. Cellular RNA from Neplanocin A-treated cells was substantially less efficient than RNA from control cells in priming cell-free influenza transcription reactions. These results suggest that the observed inhibition of influenza virus replication is due at least in part to impaired recognition of undermethylated cellular mRNA cap structures by the influenza polymerase complex.

Neplanocin A; RNA methylation inhibitor; Influenza virus; Priming of mRNA synthesis

Introduction

Neplanocin A (NPC) is a cyclopentenyl adenosine analogue which exhibits antiviral activity against a wide variety of viruses, including vaccinia virus, vesicular stomatitis virus, reovirus and measles virus [4,5a]. Concentrations of NPC which

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are effective in reducing viral replication markedly inhibit cellular *S*-adenosylhomocysteine (SAH) hydrolase [4,6]. Intracellular accumulation of SAH inhibits methyltransferases which use *S*-adenosylmethionine (SAM) as a methyl donor [11]. For some viruses which replicate within the cytoplasm, NPC treatment presumably results in the inhibition of viral methyltransferases responsible for generation of 7-methylguanosine (m^7G) residues in the mRNA cap, leading to inefficient association of viral mRNA with ribosomes [3,4].

NPC also has antiviral activity against influenza (R.K. Robins, personal communication), a negative-strand RNA virus which replicates its genome within infected cell nuclei [10]. This inhibition is not due to impairment of a viral methyltransferase, because all methylation of influenza viral mRNA is catalyzed by cellular enzymes [21]. We wished to determine whether inhibition of influenza virus replication could result from reduced cellular methyltransferase activity. It was of interest to do so because influenza virus transcription is dependent upon cleavage of capped primers from newly synthesized host mRNAs [15,22] and experiments using in vitro transcription systems indicate that efficient priming requires methylation in the 5′ cap, both at m^7G and at the 2′-*O* position of the adjacent nucleoside [2]. Thus, when viral nucleocapsids are used for in vitro transcription, mRNAs possessing the cap 1 ($m^7GpppNm$) structure are 14-fold more active in priming than those with a cap 0 (m^7GpppN) structure [2]. In addition to these cap methylations associated with the primer, influenza viral mRNAs are also methylated at internal adenosine residues (m^6A) [14].

Here, we show by high pressure liquid chromatography (HPLC) analysis of cellular RNA that NPC treatment of Chinese hamster ovary (CHO) cells results in a pronounced inhibition of both 2′-*O*-methylnucleosides in the 5′ cap and of internal m^6A residues. In these NPC-treated cells, influenza-specific mRNA accumulation is drastically reduced. We provide evidence that this inhibition of viral transcription could be the result of inefficient cap recognition by the influenza polymerase complex, because undermethylated RNA extracted from NPC-treated cells is substantially less efficient than control RNA as a source of primer for in vitro influenza transcription.

Materials and Methods

Cells and virus

The WSN strain of influenza virus was grown on MDBK cells and titered by plaque assay on MDBK cells as previously described [13]. CHO 14-10-4 [9] cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 2 mM glutamine, antibiotics and 10% fetal bovine serum.

Drug treatment and viral infection

Confluent CHO cell monolayers were incubated in DMEM with the indicated levels of NPC for 4 h at 37°C washed with phosphate-buffered saline (PBS), and

infected with 50 pfu/cell of influenza virus as previously described [23]. Cells were maintained in the indicated concentrations of NPC, except during the 1-h adsorption with virus. In all experiments, cells were incubated with NPC for a total of 8 h.

Analysis of viral proteins

Confluent cell monolayers in 16 mm multiwell dishes were incubated for 4 h with NPC and infected with influenza virus. Four hours postinfection, cells were washed, resuspended in methionine-free media (Gibco) supplemented with 10% dialyzed fetal bovine serum (Gibco) for 15 min and labelled with 50 μCi/ml of [^{35}S]methionine (NEN Research Products, 10 mCi/ml, 1100 Ci/mmol) for 45 min. Cell extracts were prepared by lysis with 2% SDS. Labelled proteins were analyzed on 15% SDS polyacrylamide gels by electrophoresis followed by autoradiography.

Isolation of RNA

Total cellular RNA. Total cellular RNA for HPLC analysis was prepared by lysing cells in 4 M guanidinium isothiocyanate and centrifugation through 5.7 M CsCl as described [19]. Polyadenylated RNA was isolated by oligo(dT)-cellulose affinity chromatography [1a].

Nuclear RNA. Detergent-washed nuclei were prepared and nuclear RNA was extracted with guanidinium isothiocyanate-hot phenol as described [23]. Polyadenylated RNA was isolated by oligo(dT)-cellulose affinity chromatography.

Cytoplasmic RNA. After nuclei were pelleted, the supernatant was extracted twice with phenol-chloroform, and once with chloroform. Polyadenylated RNA was isolated as described above.

Gel electrophoresis and blot hybridization analysis of RNA

0.25 μg of cytoplasmic polyadenylated RNA or 4 μg of nuclear RNA was fractionated by electrophoresis on 1.2% agarose/6% formaldehyde gels, transferred to Genescreen (NEN Research Products), and subjected to blot hybridization analysis as previously described [23]. Following hybridization, filters were washed, and exposed to X-ray film (Kodak XAR-5).

HPLC analysis of methylated constituents in CHO polyadenylated RNA

Monolayers of CHO cells were incubated in methionine-free medium with 0, 1, 10 or 100 μM NPC. This medium was supplemented with 20 μM adenosine and guanosine and 20 mM sodium formate to inhibit purine ring labelling [1]. After 3.75 h, actinomycin D was added to 40 ng/ml, to inhibit ribosomal RNA synthesis.

At 4 h, cells were labelled with [^{3}H]methyl methionine (ICN, 1 mCi/ml, 50 Ci/mmol), and [^{14}C]uridine (Amersham, 2 μCi/ml, 529 mCi/mmol), for 4 h. Total cellular polyadenylated RNA was prepared and digested to nucleosides and cap structures as described [1]. HPLC analysis was performed as previously described [1].

In vitro synthesis of influenza mRNA

Nuclear extracts of influenza-infected HeLa cells were prepared as described [7]. Standard 50 μl reactions contained (including components derived from added extract) 5 mM Hepes/KOH, pH 7.9, 40 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 5 mM dithiothreitol, 5 mM creatine phosphate, 2% glycerol, 1 mM ATP, CTP, GTP and 25 μM UTP, containing 30 μCi of α-[^{32}P]UTP (Amersham, 20 mCi/ml, 800 Ci/mmol) and an RNA source of primer as indicated in the text. After incubation at 31°C for 90 min, reactions were made 0.5% in SDS and 0.5 M in LiCl in 1 ml and polyadenylated RNA was purified by affinity chromatography on oligo(dT)-cellulose.

Results

Viral protein synthesis in influenza-infected, NPC-treated CHO cells

These studies were conducted in CHO cells, because NPC action and metabolism in this system have been characterized [24] and influenza replication through the stage of protein synthesis is similar to that observed in permissive cells [16]. To assess the effect of NPC on viral protein synthesis, cells were pretreated for 4 h with increasing concentrations of NPC before viral infection, using levels of NPC which had previously been shown by Glazer and Knode to inhibit cellular RNA methylation [8]. Cell extracts were prepared 4 h postinfection, so that total time of exposure to NPC was 8 h. In cells treated with 1 μM NPC, there was no effect on influenza protein synthesis, as determined by autoradiograms of [^{35}*S*]methionine-labelled proteins (Fig. 1, lanes 2–3). However, in cells pretreated with 10 μM or 100 μM NPC, there was a clearcut reduction in synthesis of viral proteins (Fig. 1, lanes 4–5). In contrast to the marked effect on viral protein synthesis, treatment of uninfected cells with 100 μM NPC for 8 h did not affect the pattern of cellular proteins synthesized in CHO cells (Fig. 1. compare lanes 1 and 6); nor was incorporation of [^{35}*S*]methionine into trichloroacetic acid (TCA)-precipitable material affected by NPC treatment (data not shown). The effect of NPC on viral protein synthesis at 4 h postinfection was not due to a shift in the time course of replication, since viral protein synthesis in NPC-treated cells was equally inhibited at later timepoints (data not shown).

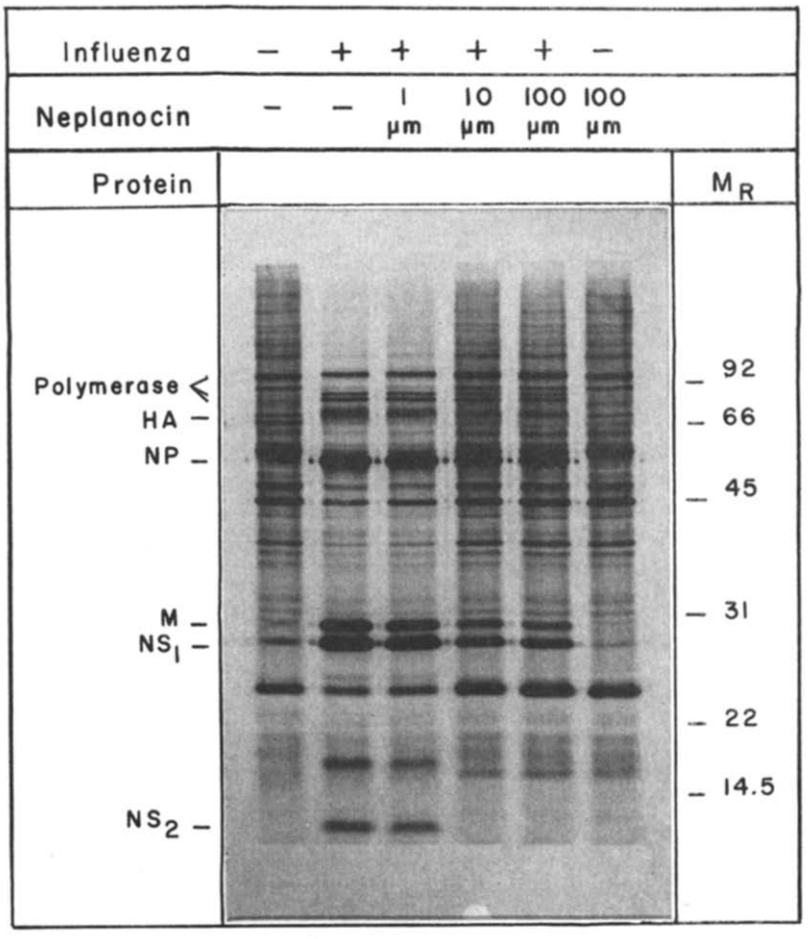

Fig. 1. Influenza virus protein synthesis in NPC-treated CHO cells. NPC treatment of CHO cells, influenza virus infection, preparation of cell extracts and analysis of [^{35}S]methionine labelled proteins are described in Materials and Methods. Individual influenza proteins were identified by comparison with the figures of Lamb and co-workers [16]. Positions of SDS/PAGE low molecular weight standards (Bio Rad) are indicated. Lane 1 = mock-infected; lane 2 = untreated, influenza-infected; lane 3 = 1 µM NPC, influenza-infected; lane 4 = 10 µM NPC, influenza-infected; lane 5 = 100 µM NPC, influenza-infected; lane 6 = 100 µM NPC, mock-infected.

Viral mRNA accumulation in influenza-infected, NPC-treated CHO cells

These results, in agreement with Saunders and co-workers [24], indicated that NPC treatment did not directly affect translation in CHO cells, and suggested that reduced influenza mRNA availability was the cause of the observed decrease in viral protein synthesis. Accordingly, we evaluated the accumulation of influenza mRNA in CHO cells pretreated with 10 µM or 100 µM NPC. Cells were pretreated for 4 h and infected with influenza virus. Cytoplasmic polyadenylated RNA

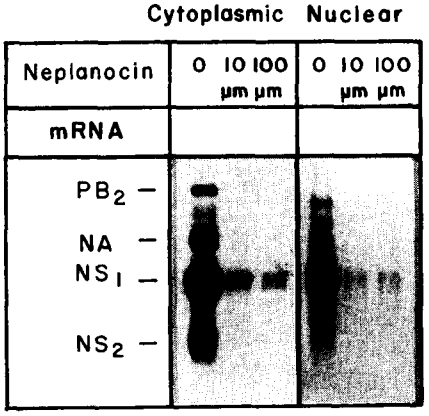

Fig. 2. Influenza virus mRNA accumulation in NPC-treated CHO cells. CHO cells were treated with NPC and infected with influenza virus as described above; RNA preparation and blot hybridization analysis are described in Materials and Methods. Hybridization probes used in these assays detect mRNAs encoding a polymerase protein (PB 2), a glycoprotein (NA), and the major nonstructural protein (NS 1), as well as a spliced alternative transcript (NS 2), which also encodes a nonstructural protein [18]. It was determined by blot hybridization analysis of cellular mRNAs actin and tubulin that equal amounts of intact RNA were loaded on each lane (data not shown). Sizes of transcripts were determined by the relative mobility of ^{32}P-labelled RNAs of known molecular weight, generated in vitro with SP-6 polymerase (NEN). In each autoradiogram, lane 1 = influenza-infected; lane 2 = influenza-infected, 10 μM NPC; lane 3 = influenza-infected, 100 μM NPC.

was prepared at 4 h postinfection and analyzed by blot hybridization for influenza transcripts encoding a polymerase protein (PB 2), a glycoprotein (NA) and the major nonstructural protein (NS 1). At both concentrations of NPC tested, influenza transcripts were barely detectable (Fig. 2). Blot hybridization analysis indicated that these concentrations of NPC did not affect steady-state levels of two cellular mRNAs, actin and tubulin (data not shown). Because experiments by Camper and co-workers in cells treated with the RNA methylation inhibitor *S*-tubericidinylhomocysteine (STH) suggested delayed cytoplasmic appearance of undermethylated mRNAs [5], it was of interest to ascertain whether there was intranuclear accumulation of influenza mRNA. Therefore, influenza mRNAs in nuclear RNA extracted from NPC-treated infected cells were assayed by blot hybridization. Pretreatment with NPC, infections and controls were identical to those described above. This analysis demonstrated a marked reduction of influenza transcripts in nuclear RNA in NPC-treated cells (Fig. 2), indicating a defect in viral mRNA synthesis or stability, rather than processing or transport.

HPLC analysis of methylation in CHO mRNA

Because these results indicated that influenza mRNA synthesis in NPC-treated CHO cells might be impaired, we determined whether the methylation state of cellular mRNAs was altered in such a way as to affect recognition of host mRNA

caps by the influenza polymerase complex. mRNA methylation in CHO cells was characterized by means of HPLC analysis as previously described [1]. This analysis provides a complete profile of methylated constituents found in eukaryotic mRNAs, including resolution of cap 1 and cap 0 structures. Concentrations of NPC and duration of exposure to NPC were those used in assays of influenza protein synthesis

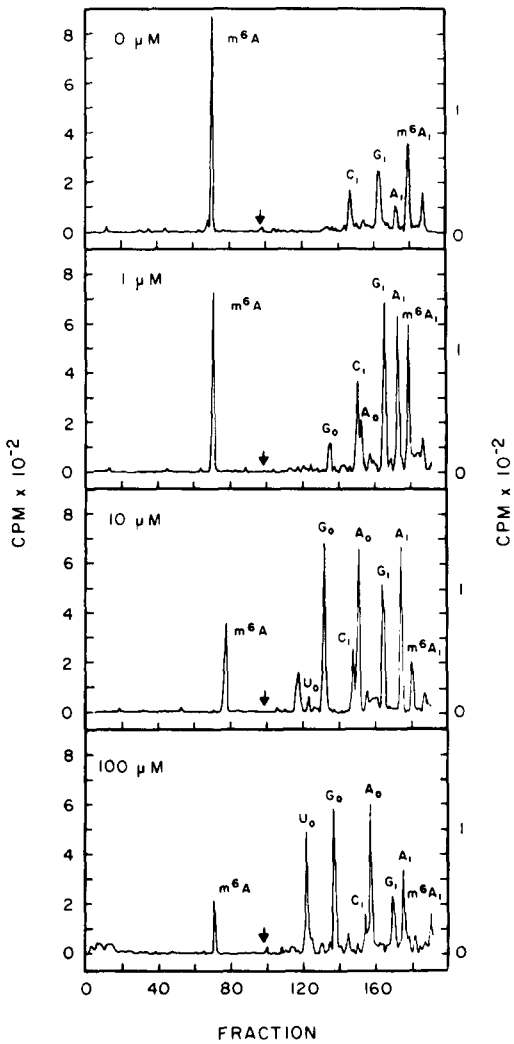

Fig. 3. HPLC analysis of the effect of NPC on mRNA methylation in CHO cells. NPC treatment, preparation of mRNA, digestion to nucleotides and cap cores and HPLC analysis are described in Materials and Methods. Peaks in fractions 100 (arrow) to 180 have been expanded 5× so that the different cap structures can more easily be identified. m^6A = N^6-methyladenosine. C_1, G_1, A_1, U_1, m^6A_1 = cap 1 structure in which there is a 2′-*O*-methylnucleoside adjacent to m^7G ($m^7GpppNm$). C_0, G_0, A_0, U_0 = cap 0 structure (m^7GpppN).

and mRNA accumulation. Incubation with these levels of NPC (1–100 μM) did not affect incorporation of [^{14}C]uridine into polyadenylated RNA (data not shown). Both methylation of internal adenosine residues (Fig. 3: compare m^{6}A peaks) and 2′-*O*-methylation within cap structures were markedly inhibited. The inhibition of 2′-*O*-methylation is revealed by a pronounced shift from predominance of cap 1 to cap 0 structures [Fig. 3: compare peaks representing cap 1 (e.g. A1) to peaks representing cap 0 (e.g. A0)]. The cap 1 fraction decreased from 92% to 73% in mRNA from cells treated with 1 μM NPC; at both 10 μM and 100 μM, the fraction fell to 37%. Total [^{3}H]methyl counts in polyadenylated RNA decreased with increasing NPC (data not shown), as a result of the inhibition of m^{6}A and 2′-*O*-methylation (Fig. 3). Despite the decreased incorporation of [^{3}H]methyl counts into 2′-*O*-methylnucleosides in cap structures (Fig. 3), the proportion of total [^{3}H]methyl counts in cap structures increased (Fig. 3). From this observation, we infer that generation of the m^{7}G portion of the cap is not affected by exposure to NPC. These results suggested that decreased efficiency of priming due to a reduction in cap 1 mRNA might account in part for the observed reduction of influenza transcripts.

Priming of in vitro influenza transcription by nuclear RNA from NPC-treated CHO cells

In order to assay the priming efficiency of RNA from NPC-treated cells, we employed nuclear extracts of influenza-infected HeLa cells as an efficient in vitro influenza transcription system. In these extracts, viral mRNA synthesis is almost entirely dependent upon the addition of added RNA as a source of primers. As exogenous primer, we prepared polyadenylated nuclear RNA from CHO cells

TABLE 1

Evaluation of RNA obtained from NPC-treated CHO cells as a source of primer for influenza transcription in vitro.

Exp.	Unprimed (background)	CHO		% decrease in priming
		–NPC	+NPC	
1	1280	8554	4608	55
2	2085	9427	5875	48
3	1490	12460	8525	36

In vitro transcription reactions and preparation of polyadenylated CHO RNA are described in Materials and Methods; equal amounts of the indicated RNA were added as primer to each reaction. Amounts of CHO nuclear RNA from NPC-treated or control cells were equalized by comparing densitometric scans of autoradiograms of RNA blots probed with both actin and tubulin cDNA. Polyadenylated nuclear RNA was added to reactions in experiments 1 and 2; polyadenylated cytoplasmic RNA was added in experiment 3. In simultaneous control reactions primed with 6 μg of globin mRNA, there were 44632 cpm in experiment 1, 38071 cpm in experiment 2, and 59674 cpm in experiment 3. Percent decrease in priming was calculated as cpm in reactions primed with RNA from NPC-treated cells divided by cpm in reactions primed with RNA from untreated cells, where each value was normalized by subtraction of background (cpm in an unprimed reaction). To assure that radioactive material faithfully reflected amounts of synthesized influenza mRNA, equal aliquots were ethanol precipitated and analyzed by gel electrophoresis and autoradiography (data not shown).

treated for 8 h with 100 μM NPC. Nuclear RNA was used because it approximates the newly-synthesized host transcripts which serve in vivo as a source of primers for the influenza polymerase complex [17], and is enriched for transcripts synthesized during maximal NPC effect. In vitro transcription reactions were primed with equal amounts of RNA from NPC-treated or control CHO cells, or previously determined optimal concentrations of globin mRNA. Negative controls were unprimed reactions. We observed a 51% decrease in transcripts synthesized in reactions primed with nuclear RNA prepared from NPC-treated cells (Table 1, experiments 1 and 2). A decrease in priming efficiency (36%) was also observed when the source of primers was cytoplasmic polyadenylated RNA from NPC-treated cells (Table 1, experiment 3). However, the reduction in priming efficiency was less, probably because of the presence of previously-synthesized, fully-methylated RNA.

These experiments indicated that priming of influenza mRNA transcription could be substantially inhibited in NPC-treated CHO cells. However, the extent of inhibition (51%) was much less than the observed inhibition of mRNA accumulation. It is not feasible to determine directly whether a decrease of 51% in mRNA synthesis could account fully for the virtual absence of amplified influenza mRNAs. However, since amplified influenza mRNA synthesis requires ongoing viral protein synthesis [25], it is possible to approximate this level of inhibition by partially inhibiting protein synthesis. Thus, influenza mRNA accumulation was assessed in CHO cells infected in the presence of sufficient cycloheximide to inhibit protein synthesis by 50% (quantitated by incorporation of [^{35}S]methionine into TCA-precipitable material). We observed a decrease of greater than 95% in accumulation of viral transcripts (data not shown). This result indicated that a partial block in influenza gene expression at an early stage of replication could essentially abolish the accumulation of amplified mRNA.

Discussion

These experiments indicate that influenza gene expression is severely and specifically restricted in NPC-treated CHO cells. At least in part, this inhibition is accounted for by the inability of newly-synthesized cellular mRNAs possessing a cap 0 structure to function as a source of primer for influenza mRNA synthesis (Table 1). Despite their failure to function efficiently to prime influenza transcription, these same undermethylated cellular mRNAs appear capable of serving as effective templates for cellular protein synthesis (Fig. 1, lane 6), a result which is consistent with the observations of Kaehler and co-workers in Novikoff hepatoma cells [12]. It is likely that the 51% impairment in priming which we determined in vitro is a minimum estimate of the decrease which occurs during infection of intact NPC-treated cells, because influenza transcription is primed in vivo exclusively by newly-synthesized host transcripts. The polyadenylated nuclear RNA extracted from NPC-treated cells used as primer for in vitro transcription was probably contaminated with previously synthesized, fully-methylated RNA. The residual priming activity

in nuclear RNA isolated from NPC-treated CHO cells, approximately 49% of control (Table 1), is consistent with our determination that approximately 40% of RNA in treated cells possesses a cap 1 structure (Fig. 3). Additionally, RNA with a cap 0 structure retains some priming activity [2].

Our results in cycloheximide-treated cells support the notion that partial impairment of priming due to loss of cap 1 mRNAs in NPC-treated cells could explain a profound reduction in influenza mRNA accumulation. However, we are not able to exclude the possibility that other consequences of NPC treatment could contribute to its antiviral effects in this system. In this regard, internal m^6A methylation in NPC-treated cells was markedly diminished. We have recently determined that distribution and localization of m^6A residues in influenza mRNAs suggest a potential biologic role of m^6A in viral mRNA metabolism [20]. Additionally, Krug and co-workers have provided evidence that the portion of influenza mRNAs derived from cellular transcripts contains internal m^6A, and suggested that the presence of these residues might enhance the efficiency of priming [15]. Thus, a decrease in m^6A residues could also contribute to the observed inhibition of influenza replication. A further possibility is suggested by the observation that in CHO cells, NPC is metabolized to the triphosphate (NPC-TP), a potential substrate for viral or cellular RNA polymerases [24]. However, Glazer and Knode monitored the metabolism of NPC-TP in HT-29 cells, in which nanomolar amounts are formed, and observed incorporation into ribosomal and transfer RNA to be 0.001%, and incorporation into mRNA to be nil [8].

In conclusion, our results provide evidence in vivo consistent with requirements previously described in vitro for efficient mRNA cap recognition by the influenza polymerase complex. Accordingly, the unusual mechanism employed by influenza virus to prime mRNA synthesis may provide a selective target for interrupting viral replication.

Acknowledgements

We thank Patricia A. Maroney for helpful suggestions and critical review of the manuscript, and Ronald T. Borchardt of the University of Kansas and Robert I. Glazer of the National Cancer Institute for providing Neplanocin A.

This investigation was supported by National Science Foundation grant number PCM 84-02121 to T.W.N. and American Cancer Society grant number CA-31810 to F.M.R. R.M.R. is supported by a Physician's Research Training award of the American Cancer Society. T.W.N. is a recipient of the Presidential Young Investigator award of the National Science Foundation.

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