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Measles virus-specific dsRNAs are targets for unwinding/modifying activity in neural cells *in vitro*

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Biased hypermutation events found predominantly in the matrix gene of measles virus isolated from persistent human CNS infections have been attributed to the action of a cellular unwinding/modifying activity (UMA). To define the level and distribution of this activity in brain cells, fractionated extracts were prepared from the nuclei and cytoplasm of human glioblastoma (D-54, U-251) and neuroblastoma (IMR-32, SKN-MC) cells and analyzed for their ability to modify synthetic dsRNAs specific for the measles virus (MV) matrix (M) gene. On a quantitative basis we could show that the activity localized to both the nuclear and cytoplasmic compartments of both cell types analyzed independent of cell proliferation. The presence of significant levels of UMA in the cytoplasm of human brain cells following growth arrestment *in vitro* with retinoic acid supports the interpretation that UMA may contribute to the attenuation of MV gene functions during the primary infection of brain cells, thereby supporting the establishment of virus persistence.

Keywords: measles virus; unwinding/modifying activity

Introduction

As rare, late complications of acute measles, persistent measles virus (MV) infections of the human central nervous system (CNS) develop as subacute sclerosing panencephalitis (SSPE) and measles inclusion body encephalitis (MIBE). Virologically, these conditions are characterized by a highly restricted MV gene expression leading to strict cell associated viral replication and a complete absence of infectious virus particles (reviewed in Schneider-Schaulies and ter Meulen, 1992). Whereas nucleocapsid protein (N) and phosphoprotein (P), as components of the viral ribonucleotide particle (RNP) are easily detected in neurons and glial cells in autopsy material, the expression of the matrix (M), fusion (F) and hemagglutinin (H) envelope proteins is typically confined to a few infected cells or is completely absent (Liebert *et al*, 1986). In addition to very low frequencies of the corresponding mRNSs, some of these reading frames have been shown to be loaded with mutations that affect or abolish the expression of the corresponding gene products (for a review see Billeter *et al*, 1994).

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Extensive sequence analyses performed on MV structural genes isolated from persistent brain infections or tissue culture cells indicated that basically two types of mutations can be distinguished. Point mutations, which are typically found to occur in any viral gene and are linked in part to the genetic variability present in co-circulating lineages of MV (Baczko *et al*, 1992; Taylor *et al*, 1991), and hypermutations involving simultaneous clustered transitions of several uridine (U) to cytidine (C), or, less frequently, adenosine (A) to guanosine (G) residues, specified in the plus strand sense. This latter type of mutation has been encountered predominantly in the MV M genes of SSPE and MIBE cases and persistently infected tissue culture cells (Cattaneo *et al*, 1988a,b; Ayata *et al*, 1989; Cattaneo *et al*, 1989; Wong *et al*, 1989; Baczko *et al*, 1989) and was ascribed to the activity of a double stranded (ds) RNA dependent unwinding/modifying activity (UMA) intrinsic to the host cell (Bass and Weintraub, 1988; Cattaneo *et al*, 1988a, b; Bass et al, 1989). The model predicts that A residues would be desaminated to yield inosine (I) in MV specific dsRNAs during transcription. Subsequently, within the first round of replication, the modified I residues would base pair with C replacing the primary A/U pairing.

The potential pathogenetic importance of this cellular activity in silencing MV gene functions has recently been shown in a study characterizing the evolvement of hypermutated sequences within different brain areas of a case of SSPE (Baczko *et al,* 1993). Moreover, hypermutated viral genes have been observed in other viral infections (O'Hara *et al,* 1984; Murphy *et al,* 1991; Antic *et al,* 1992) suggesting a host mechanism for the downregulation of viral gene expression.

Although this enzyme remains to be characterized, its activity could be detected in a variety of mammalian tissue culture cells without an apparent template specificity (Wagner and Nishikura, 1988; Wagner *et al,* 1990; Nishikura *et al,* 1991). The UMA has been localized to the cell nucleus and is dependent on the cell cycle (Bass and Weintraub, 1987; Wagner and Nishikura, 1988) usually being extremely low under growth arresting conditions.

In a previous study, a MV M specific subgenic fragment has been successfully modified in nuclear extracts of a human neuroblastoma cell line (IMR-32) (Rataul *et al,* 1992). However, since MV replicates only in the cytoplasm of an infected cell it is unclear how a nuclear activity could affect MV gene functions. We therefore reinvestigated the UMA in human neural cells. In a comparative analaysis using fractionated extracts of two neuroblastoma (IMR-32, SKN-MC) and two glioblastoma (D-54, U-251) cell lines the activity could be detected in both the nucleus and cytoplasm to varying degree under standard growth conditions. No significant loss of UMA was observed regardless of whether growth arrestment had been effected after retinoic acid treatment of the cells. These findings indicate that in brain cells significant levels of UMA are present in the cytoplasm independent of proliferation that may contribute to the primary protection of the host cell from a lytic infection.

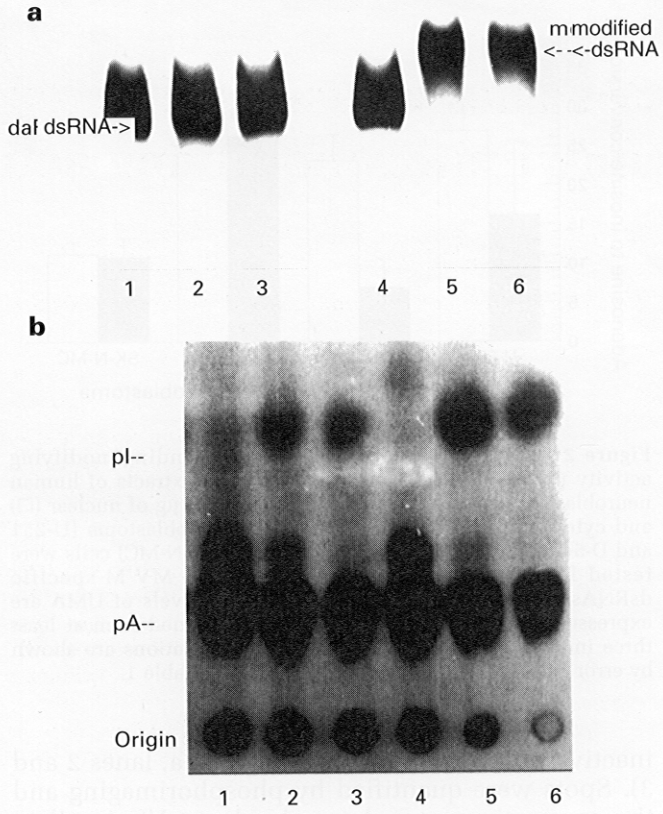

Figure 1 Unwinding/modifying activity visualized by gel shift and thin layer chromatography (TLC). Subgenic MV M-specific dsRNAs (Xho2/EcoRI, nt 3445-3972) were incubated for 3 h each with 60 µg cytoplasmic (each lane 1–3) and nuclear (each lane 4–6) extracts prepared from human glioblastoma cells (D-54) and, for control, with the corresponding heat inactivated extracts (each lanes 1 and 4). Reaction products were separated by a native PAGE (a) or following nuclease P1-digestion into mononucleotides, by TLC (b). The positions of the mononucleotides pA and pI are indicated. For this particular experiment, the A/I conversion rates were 5.8% and 6.4% (lanes 2 and 3) for the cytoplasmic extracts and 20.7% and 28.3% (lanes 5 and 6) for the nuclear extracts. Spots visible above the pA signal in (b) (lane 1 and 4) are artefacts.

Results

Unwinding/modifying activity in neural tissue culture cells

To determine the intracellular localization of the UMA and its specificity for MV templates, nuclear and cytoplasmic extracts were prepared from human glioblastoma (D-54) cells. Complementary subgenic MV M-specific RNAs were synthesized *in vitro* in the presence of ^{32}P-ATP, hybridized to generate dsRNAs that were, after purification, used as templates. For control, the individual reaction products were tested for their sensitivity to RNAse treatment (data not shown). Following titration of the cell extracts and time courses (data not shown), 60 µg of cell extract and 3 h incubation at 37°C were used as standard conditions for all subsequent experiments.

Using the 527 bp subgenic MV M-specific dsRNA (derived from the 5' end of the M mRNA), a characteristic conformational shift, indicated by the occurrence of higher migrating reaction products due to UMA, could be observed in two independent nuclear (Figure 1a, lanes 5 and 6) but not in cytoplasmic extracts (Figure 1a, lanes 2 and 3) of D-54 cells. Also typically, the activity could be eliminated by heat inactivation of the extract prior to incubation (Figure 1a, lanes 1 and 4).

To quantitate the levels of UMA, the conversion rates of adenosine (A) to inosine (I) were determined following digestion of the reaction products into mononucleotides and subsequent separation by thin layer chromatography (Figure 1b). Due to the higher sensitivity of this assay, UMA could also be detected in the cytoplasmic extracts (Figure 1b, lanes 2 and 3) that were found to be essentially

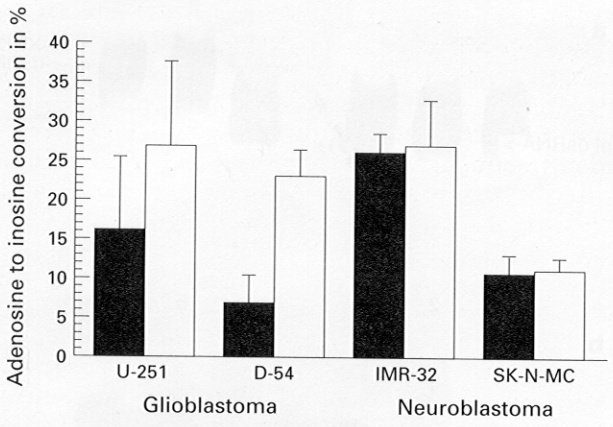

Figure 2 Comparative analysis of the unwinding/modifying activity present in nuclear and cytoplasmic extracts of human neuroblastoma and glioblastoma cells. Each 60 µg of nuclear (□) and cytoplasmic (■) extracts from human glioblastoma (U-251 and D-54) and neuroblastoma (IMR-32 and SK-N-MC) cells were tested for their ability to modify subgenic MV M-specific dsRNAs (Xho2/EcorRI, nt 3445-3972). The levels of UMA are expressed as mean A/I conversion rates obtained from at least three independent experiments. Standard deviations are shown by error bars. Numeric values are indicated in Table 1.

inactive by gel shift analysis (Figure 1a, lanes 2 and 3). Spots were quantified by phosphorimaging and the conversion rates determined as pI/(pA+pI) to 5.8% and 6.4% in cytoplasmic and 20.7% and 28.3% in nuclear extracts of D-54 cells in this experiment. It is important to note that in ascertaining the statistical relevance of our results, subsequent experiments were performed with at least two to three independent preparations of each extract and reproduced at least twice using the same extract preparation.

Cell type specific and intracellular distribution of the unwinding/modifying activity in neural cells

Nuclear and cytoplasmic extracts of the two human neuroblastoma (IMR-32, SKN-MC) and glioblastoma (D-54, U-251) cell lines were comparatively analyzed for their ability to modify the subgenic MV M-specific dsRNAs. In nuclear extracts, comparable levels of UMA could be determined for all cell lines with the exception of SKN-MC cells (Figure 2, open columns). As detailed above (Figure 1b), considerable amounts of UMA were consistently detected in the cytoplasmic fractions of all cell lines investigated. For both glioblastoma cell lines, the overall levels of cytoplasmic activity (Figure 2, filled columns) were lower than those found in the nuclear fractions (Figure 2, Table 1). Cytoplasmic extracts prepared from neuroblastoma cells, however, revealed essentially the same levels of activity as determined for their nuclear counterparts (Figure 2, Table 1). Thus, UMA was not only confined to the nucleus but was also detected in the cytoplasmic compartment in cells of neural and neuronal origin.

Proliferation dependence of the unwinding/modifying activity in neural cells

As our results were contradictory to previous findings with IMR-32 cells (Rataul *et al*, 1992), we investigated whether the UMA detected in the cytoplasmic fractions of neural cells would reflect the leakage of the high nuclear activity as a consequence of cell division during proliferation. This is particularly important as the proliferation rate of differentiated brain cells *in vivo* is particularly low, and a reduction in UMA has been described for NIH 3T3 cells after growth arrestment by serum starvation (Wagner and Nishikura, 1988). Thus, the proliferation rates of our cell lines were determined using

Table 1 UMA in nuclear and cytoplasmic extracts of human neural and neuronal cell lines

	UMA (A/I conversion in %)[a]					
	Standard growth conditions			*100µ M retinoic acid*		
dsRNA	*MF*		*ME*	*MF*		*ME*
Cell line	n[b]	c[b]	c	n	c	c
U-251	27 (10.6)	16.3 (9.2)	6.8 (2.9)	19.6 (7.9)	17.3 (8.1)	29 (0.2)
D-54	23.4 (3.3)	6.9 (3.7)	4.2 (0.5)	22.8 (8.6)	11.9 (5)	n.d.
IMR-32	27 (5.8)	26.4 (2.4)	33 (4.5)	28.4 (6.1)	24.4 (4.2)	33.4 (1.2)
SKN-MC	11.4 (1.6)	11 (2.3)	11.1 (10.9)	24.6 (5.5)	20.3 (6.4)	n.d.

UMA present in nuclear and cytoplasmic extracts of human glioblastoma (U-251, D-54) and neuroblastoma cells (IMR-32, SKN-MC) was analyzed using MV M-specific dsRNAs covering either a fragment (MF) or the entire gene (ME). UMAs are expressed as mean values of at least three independent experiments as A/I conversion rates in %, numbers in brackets indicate the standard deviations.
[a] UMA determined as A/I conversion rate in %
[b] n means nuclear extract, c cytoplasmic extract
n.d. means not determined

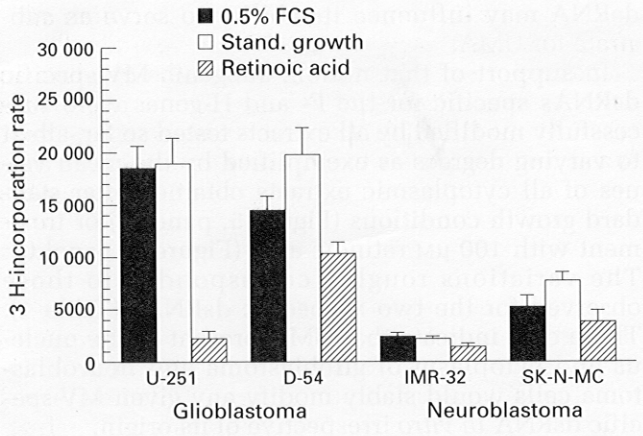

Figure 3 Analysis of the proliferation rates of human glioblastoma and neuroblastoma cells cultivated under different growth conditions. Glioblastoma (U-251 and D-54) and neuroblastoma (IMR-32 and SKN-MC) cells were cultivated under low serum (0.5% FCS) (■), standard growth conditions (5% FCS) (□) and in the presence of 100 μM retinoic acid (5% FCS) (hatched columns) for 24 h as described (Materials and methods). ^{3}Hdt incorporation into cellular DNA as a measure of cell proliferation was determined by scintillation counting (Pharmacia, Munzingen Germany).

different culture conditions including serum starvation or treatment with the morphogen retinoic acid for 24 hours (Figure 3). Most surprisingly, serum starvation did not (for U-251 and IMR-32 cells) or just merely (for D-54 and SKN-MC cells) affect cell division (Figure 3). Morphological alterations, probably indicating differentiation, could be observed upon treatment with retinoic acid after 24 h in all cells lines (not shown). Although not completely arrested, at least three cell lines proved to be sensitive to the antiproliferative effect of retinoic acid (up to 90% reduction for U-251 cells), whereas the overall low proliferation rate of IMR-32 cells was just merely affected (Figure 3). It should be noted, however, that although IMR-32 cells revealed a generally low proliferative activity under all culture conditions applied, cytoplasmic extracts of these cells consistently revealed comparatively high levels of UMA (Figure 2) indicating that UMA in neural cells was probably independent of cell proliferation.

To confirm this hypothesis, UMAs present in glioblastoma and neuroblastoma cells grown under standard conditions (panel B) or growth inhibited after treatment with retinoic acid (panel C) were comparatively analyzed for the subgenic MV M-specific dsRNAs (Figure 4, Table 1).

With the exception of SKN-MC cells, no significant stimulation of the nuclear UMA could be detected after treatment with retinoic acid (Figure 4,

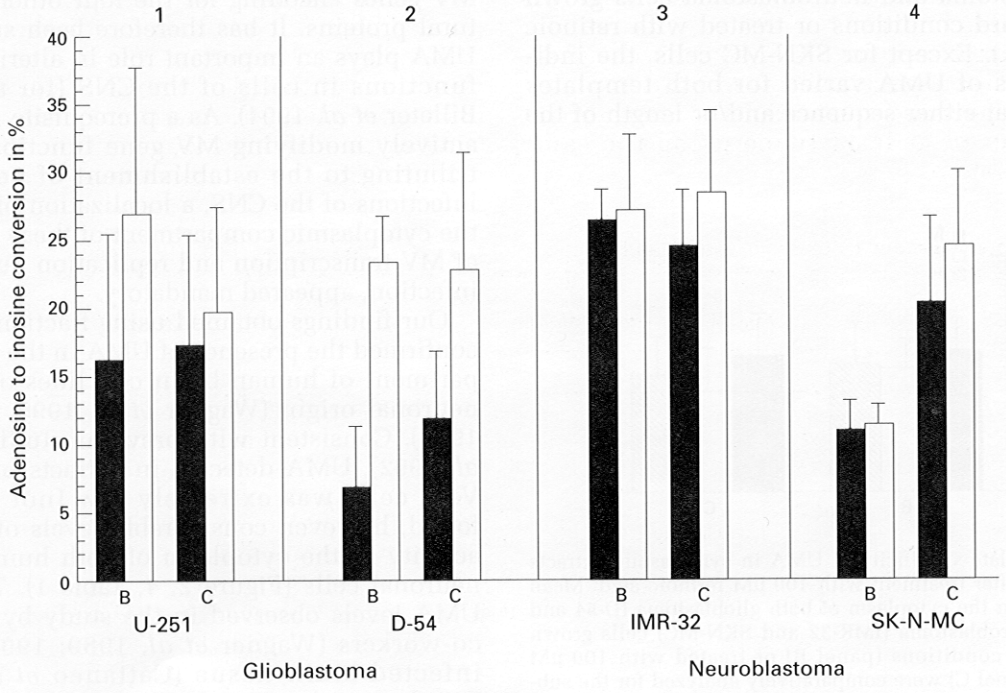

Figure 4 Influence of proliferative inhibition after treatment with 100 μM retinoic acid on UMA present in nuclear and cytoplasmic extracts of human neural and neuronal cells. Subgenic MV M-specific dsRNAs (Xho2/EcoRI, nt 3445-3972) were incubated with cytoplasmic (■) or nuclear extracts (□) of glioblastoma (U-251 and D-54, panels 1 and 2) and neuroblastoma (IMR-32 and SK-N-MC, panels 3 and 4) cells grown under standard culture conditions (5% FCS) (B) or in the presence of 100 μM retinoic acid (5% FCS + 100 μM retinoic acid) (c). Each bar represents the mean value of at least three independent experiments. Standard deviations are represented by error bars.

panels 1–4). Despite growth arrestment, however, levels of UMA detected in the cytoplasm of both glioblastoma and neuroblastoma cells were not reduced after retinoic acid mediated growth inhibition (Figure 4, panels 1 and 4, Table 1). No statistically significant proliferation dependent differences could consistently be determined for the UMA present in cytoplasmic extracts of U-251 (0,299), IMR-32 (0,152) and SKN-MC (0,2607) cells as confirmed by double *t*-test, whereas for D-54 cells a significance level of 0,02 was defined (values below 0,05 were considered as significant). These results support our hypothesis that high levels of UMA are mounted in the cytoplasm of brain cells independent of their proliferative activity.

Template specificity of the unwinding/modifying activity in cytoplasmic extracts of differentiated neural cells

Most hypermutation events defined in MV-specific sequences from autopsy material of patients with persistent brain infections have been encountered within the M gene. We therefore investigated whether the UMA detected in the cytoplasm of neural and neuronal cells would reveal any preference for modifying M-gene specific dsRNAs *in vitro*. Synthetic dsRNAs covering the entire length of the MV Edmonston M-gene were found to serve as suitable templates for the UMA present in nuclear (not shown) and the cytoplasmic extracts of both glioblastoma and neuroblastoma cells grown under standard conditions or treated with retinoic acid (Table 1). Except for SKN-MC cells, the individual levels of UMA varied for both templates indicating that either sequence and/or length of the dsRNA may influence its ability to serve as substrate for UMA.

In support of that notion, subgenic MV-specific dsRNAs specific for the F- and H-genes were successfully modified by all extracts tested so far, albeit to varying degrees as exemplified by the mean values of all cytoplasmic extracts obtained after standard growth conditions (Figure 5, panel B) or treatment with 100 µM retinoic acid (Figure 5, panel C). The variations roughly corresponded to those observed for the two M-specific dsRNAs (Table 1). These data indicate that UMA present in the nucleus and cytoplasm of glioblastoma and neuroblastoma cells would stably modify any given MV-specific dsRNA *in vitro* irrespective of its origin.

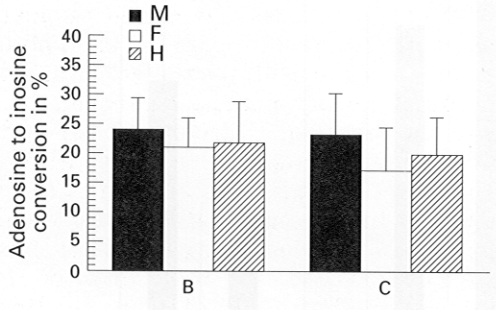

Figure 5 Template specificity of UMA in cytoplasmic extracts of neural cells after treatment with 100 µM retinoic acid. Mean levels of UMA in the cytoplasm of both glioblastoma (D-54 and U-251) and neuroblastoma (IMR-32 and SKN-MC) cells grown under standard conditions (panel B) or treated with 100 µM retinoic acid (panel C) were comparatively analyzed for the subgenic MV-M-(XhoII/EcoRI, nt 3445-3972) (■), MV-F-(Taq/DdeI nt 6925-7247) (□) and MV-H-specific (Bgl II/AvaI nt 7671-8458) (□) dsRNAs. The values indicated in this graph for each dsRNA species represent mean values including cytoplasmic extracts of both neuroblastoma and glioblastoma cells. Standard deviations of at least three independent experiments are represented by the error bars.

Discussion

It has been suggested that UMA irreversibly modifies dsRNA structures that are exceptionally and aberrantly formed during transcription and replication of negative stranded RNA viruses (including MV) involving mostly the negative stranded viral genome and a viral mRNA (Bass *et al*, 1989). Following identification of UMA as responsible for hypermutation of the MV M gene in a case of MIBE (Cattaneo *et al*, 1988a) and, later on, in other MV genes isolated from persistent MVs mostly within the M gene sequences, less extensive biased hypermutation events have been encountered within the MV genes encoding for the four other major structural proteins. It has therefore been suggested that UMA plays an important role in altering viral gene functions in cells of the CNS (for a review see Billeter *et al*, 1994). As a prerequisite for its role in actively modifying MV gene functions thus contributing to the establishment of persistent MV infections of the CNS, a localization of the UMA to the cytoplasmic compartment of these cells, the site of MV transcription and replication during primary infection, appeared mandatory.

Our findings obtained using fractionated extracts confirmed the presence of UMA in the nuclear compartment of human brain cell lines of neural and neuronal origin (Wagner *et al*, 1990; Rataul *et al*, 1992). Consistent with previous studies (Rataul *et al*, 1992), UMA detected in extracts prepared from Vero cells was extremely low (not shown). We found, however, considerable levels of the enzyme activity in the cytoplasm of both human glial and neuronal cells (Figure 2, 4, Table 1). The maximal UMA levels observed in the study by Wagner and co-workers (Wagner *et al*, 1989; 1990) or in MV infected brain tissue (Cattaneo *et al*, 1988a,b; Baczko *et al*, 1993) leading up to 50% of total residue exchange, were not detectable in our *in vitro* studies. This lower UMA activity is most likely due to maximal enzyme activities not being achieved under our experimental conditions as the base modification rates could be enhanced upon

further addition of active cell extract during the incubation (not shown). Moreover, the high mutation rates observed *in vivo* most likely result from several independent consecutive hypermutation events at the same template. This has been convincingly documented for M gene sequences isolated from the brain of one patient with SSPE that originated from at least four consecutive hypermutation events (Baczko *et al*, 1993). Interestingly, one of these sequences, already bearing hypermutations, was less efficiently modified compared to the MV ED sequence when used in our *in vitro* assay (data not shown).

Evidence for a cell-cycle dependent regulation of the activity has been obtained for mouse 3T3 cells (Wagner and Nishikura, 1988). The activity was low in quiescent cells grown under low serum conditions, but increased when the cells were stimulated to renew growth by serum. Using the same experimental approach, a moderate inhibition of proliferation could be observed in three of our four cell lines (Figure 3). UMAs present in extracts prepared from our cells after low serum treatment roughly corresponded to those found after normal standard serum conditions (not shown). As proliferation rates have not been indicated in the study by Wagner and co-workers (Wagner and Nishikura, 1988), it remains unclear whether the discrepancy observed might be related to the experimental conditions or to the different cell types used. Our cell lines (except probably IMR-32), however, proved to be sensitive to proliferative inhibition in the presence of retinoic acid, although under these conditions growth arrestment was also far from being complete (Figure 3). In spite of the proliferative inhibition observed no reduction of UMA was observed in the cytoplasmic extracts indicating that cytoplasmic UMA was independent of cellular proliferation in our cell systems (Table 1, Figure 4). This hypothesis was further substantiated by the results obtained with IMR-32 cells that revealed high UMAs in both nuclear and cytoplasmic extracts and, at the same time, the comparatively lowest proliferation rates for all experimental conditions tested (Figure 3). Besides arresting cell proliferation, retinoic acid has been described as a common morphogen promoting differentiation of certain cell lines including IMR-32 cells *in vitro* (Haussler *et al*, 1983). In our experiments, morphological changes of all cell lines following treatment with retinoic acid could be observed strongly suggesting cellular differentiation. In this particular study, attempts to identify the induction of differentiation-dependent antigens have not been performed. An earlier study revealed that at least mRNAs indicative for cellular differentiation could be induced in D-54 and U-251 cells after treatment with papaverine (Schneider-Schaulies *et al*, 1993). Extracts prepared from both cell types (neural and neuronal) after treatment with compounds raising the intracellular cAMP levels such as 8-(4-chlorophenylthio)-adenosin 3′-5′-cyclic monophosphate (8-CPT-cAMP) and papaverine confirmed the obvious requirement to maintain high levels of the UMA in either neural and neuronal cell lines after treatment with differentiating agents (not shown).

In contrast to our findings, high levels of UMA have been found in nuclear, but not in cytoplasmic extracts of the human neuroblastoma cell line IMR-32, (Rataul *et al*, 1992). The reasons for this obvious discrepancy are not clear as subgenic MV Edmonston strain M-specific dsRNAs of similar length, but different localization within the M gene, have been used in both studies. It is not very likely that this discrepancy could be attributed to the different sequences used as no particular sequence requirements for UMA have consistently been found. Some evidence has been provided for 5′ neighbor preference of A (or T) of the modified A residue and no apparent influence of the 3′ nucleotide (Kimelman and Kirschner, 1989). In another study performed with frog oocyte extracts, a certain preference for the modification of A residues 5′ to G or C residues has been described (Wagner *et al*, 1989). In addition, the subgenic M-specific fragments used in both studies were overlapping and, moreover, the entire sequence of the M gene has been found to serve as suitable template for UMA with any extract tested including the cytoplasmic extract of IMR-32 cells (Table 1). As the levels of UMA found in the cytoplasm of IMR-32 cells were comparatively high (Table 1, Figure 4), the higher sensitivity of the base modification assay used for our experiments compared to the gel shift analyses applied in the previous study (Rataul *et al*, 1992) is not likely to account for the discrepancy observed. It does also appear unlikely that UMA detected in the cytoplasmic compartment in our study merely reflects leakage of the nuclear compartment during extract preparation as in both studies basically identical protocols were used for the preparation of the cell extracts. Moreover, the distribution of the levels of UMA in nuclear and cytoplasmic compartments was basically different in neuroblastoma and glioblastoma cells (Figure 2) which would imply a generally higher fragility of the nuclei of neuroblastoma cells. This, however, could not be confirmed by the release of Hoechst 33258 dye from the nuclei of each cell line after lysolecithin treatment (see Materials and methods). Thus, the usage of different subclones of IMR-32 cells in both studies may provide the most probable explanation at present.

Although in our study any given MV-specific dsRNA proved to be a suitable target for UMA in brain cells *in vitro*, hypermutated sequences characterized so far have mainly been found in the M genes of persistent MVs (Billeter *et al*, 1994). Most probably, this obvious bias may stress the dispensability of the corresponding gene product or even

the selective advantage of viruses harboring mutated M proteins during persistent infections (Baczko *et al*, 1993) rather than indicating a template specificity of the enzyme *in vivo*.

Based on the more frequent occurrence of hypermutated sequences in persistent infections *in vivo* and our results obtained *in vitro*, a role for the unwinding/modifying activity in supporting the establishment of persistent MV infections in the human CNS can be anticipated. In the course of lytic infections in non-neural cells, modified genomes are probably not passaged and are eliminated due to competing vital genomes. In brain cells, however, additional host cell dependent transcriptional and translational restrictions imposed on MV gene expression have been described (reviewed in Schneider-Schaulies and ter Meulen, 1992) that may support the attentuation of MV gene functions as provided by active modification of MV genomic sequences.

Materials and methods

Cells and culture conditions

Human glioblastoma (D-54 and U-251, provided by D Bigner (Bigner *et al*, 1981)) and neuroblastoma (IMR-32 and SKN-MC, purchased from ATCC) cells were kept in minimal essential medium (MEM) supplemented with 5% heat inactivated fetal calf serum (FCS), glutamine, penicillin (100 U/ml)/streptomycin (100 mg ml^{-1}). For differentiation, the medium was supplemented with 100 µM retinoic acid for 24 h.

Proliferation assay

Proliferation rates were determined in triplicate assays following standard procedures for all cell lines and culture conditions indicated. Cells were pulse-labeled (0.5 mCi ml^{-1} ^{3}H-thymidine) and harvested 18 h later. ^{3}HdT incorporation was determined by liquid scintillation counting in a β-Plate (Pharmacia) and proliferation rates were calculated.

Preparation of cell extracts

Stability of the cell nuclei was analyzed after staining of the cells with Hoechst 33258 dye (Frankfurt, Germany) following standard procedures and subsequent incubation of either intact cells or separated nuclei with increasing concentrations of lysolecithin (50 µg/ml to 1mg/ml in phosphate buffered saline) at 4°C for 5 min. Extracts were prepared essentially as described (Weil *et al*, 1979; Dignam and Lebovitz, 1983). Briefly, cells were passaged or, when indicated, treated with 100 µM retinoic acid, and extracts prepared 24 h later by scraping the cells into ice-cold phosphate buffered saline (PBS). After centrifugation, cells were swollen for 10 min on ice in five packed cell volumes (PCV) of buffer A (10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT), collected by centrifugation, resuspended in two PCV of buffer A and homogenized. The solution was centrifuged at 2500 rpm and the supernatant used to prepare cytoplasmic extracts (see below). The pelleted nuclei were resuspended in buffer C (20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 25% (v/v) glycerol) and gently mixed. After centrifugation of the suspension for 30 min at 15.00 rpm, the clear supernatant was dialyzed against 50 vol buffer D (20 mM Hepes, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF 0.5 mM DTT, 20% (v/v) glycerol) for 5 h and immediately frozen in liquid nitrogen and stored as aliquots at -70°C.

For the preparation of cytoplasmic extracts, the supernatant of the low speed centrifugation was mixed with 0.11 vol buffer B (300 mM Hepes pH 7.9, 1.4 M KCl, 30 mM $MgCl_2$) and centrifuged for 60 min at 100 000 g. The supernatant was dialyzed and stored as described above. Protein concentrations were determined using a standard procedure (Sigma). On average, 1.0 to 1.5 mg total protein was obtained from 2×10^7 cells.

Preparation and purification of dsRNA templates

Recombinant pGem-1 and 4 plasmids containing subgenic fragments of the MV M (Xho2/EcoRI nt 3445-3972), F (TaqI/DdeI nt 6925-7247) and H (Bg12/AvaI nt 7672-8458) specific cDNAs were described previously (Cattaneo *et al*, 1987). Equal amounts of complementary ^{32}P-ATP labeled transcripts, obtained by *in vitro* transcription of linearized plasmid DNAs, were hybridized for 20 h at 45°C in formamide binding buffer (40 mM Hepes, pH 6.8, 400 mM NaCl, 1 mM EDTA, 80% deionized formamide). Hybrids were trimmed by nuclease S1 digestion (200 u ml^{-1} in 250 mM NaCl, 30 mM NaOAc pH 4.5, 1 mM $ZnSO_4$), purified by phenol extraction and recovered by subsequent precipitation prior to incubation with cell extract.

Unwinding assay and analysis

The unwinding reaction was performed as described previously (Bass and Weintraub, 1987, 1988; Wagner and Nishikura, 1988) in a final volume of 50 µ TGKED (50 mM Tris-HCl, pH 7.8, 25% glycerol, 0.5 mM KCl, 0.1 mM EDTA, 0.5 mM DTT) containing ^{32}P-ATP-labeled dsRNA and 60 µg cell extract at 37°C. Heat inactivation of an aliquot of each extract as performed at 60°C for 10 min. For subsequent gel shift analysis, reaction products were deproteinized by proteinase K digestion (50 mg ml^{-1} Proteinase K in 5 mM EDTA, 50 mM Tris pH 7.5, 0.5% SDS, 30 min 37°), phenol extracted, precipitated and separated on native 4% PAGE. For the base modification assay, the reaction products were digested twice with nuclease P1 (500 u ml^{-1} nuclease P1 in 10 mM Tris pH 7.4, 1 mM EDTA) for 45 min at 45° and subsequent addition of the same

constituents and further digestion for 45 min at 60°) and directly analyzed by one dimensional thin layer chromatography (TLC). As solvent system, 100 mM sodium phosphate pH 6.8/ammoniumsulfate/1-propanol, 100:60:2 (v/w/v) was used (Silberklang *et al*, 1979). Quantitative analyses were performed by phosphorimaging (PhosphorImager, Molecular Dynamics, Krefeld, Germany). Conversion rates were determined as $(I/(A+I)) \times 100(\%)$.

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