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The Cytotoxicity of the Parvovirus Minute Virus of Mice Nonstructural Protein NS1 Is Related to Changes in the Synthesis and Phosphorylation of Cell Proteins

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Autonomous parvoviruses exert lytic and cytostatic effects believed to contribute to their antineoplastic activity. Studies with inducible clones have demonstrated a direct involvement of parvovirus nonstructural proteins (NS) in oncolysis. Human and rat fibroblasts have been stably transfected with MVM(p) (minute virus of mice prototype strain) NS genes cloned under the control of a hormone-inducible promoter. Dexamethasone-induced synthesis of the NS proteins in sensitive transformed cells results in cell killing within a few days. From these sensitive cell lines have been isolated some NS-resistant clones that also prove resistant to MVM(p) infection, suggesting that cell factors modulate NS cytotoxicity. We have previously reported that factors involved in cell cycle regulation may contribute to this modulation, since NS toxicity requires cell proliferation and correlates with a cell cycle perturbation leading to an arrest in phase S/G_2. In addition to its role in cytotoxicity, NS1 can regulate transcription driven by parvovirus and nonparvovirus promoters. Since phosphorylation is a critical event in controlling the activity of many proteins, notably transcription factors and cell cycle-regulated proteins, we have examined the effect of NS1 on the synthesis and phosphorylation of cell proteins. Our results indicate that NS1 interferes, within 7 h of induction, with phosphorylation of a protein of about 14 kDa (p14). Cell synchronization has enabled us to show that phosphorylation of this protein occurs in early S phase and is prevented when NS1 is induced. This early effect of NS1 on p14 phosphorylation may be directly linked to cytotoxicity and is probably related to the previously reported inhibition of cell DNA synthesis. Late in the induction period (24 h), NS1 also alters the synthesis of a 50-kDa protein and a 35-kDa protein (p50 and p35, respectively). Microsequencing of p35 reveals sequence homology with β-tubulin. These effects of NS1, observed only in NS1-sensitive cell lines, may be related to the protein's cytotoxicity.

Parvoviruses are small, single-stranded DNA viruses without envelopes. They depend on exogenous factors to complete their life cycle. In the case of autonomous parvoviruses {e.g., the minute virus of mice prototype strain [MVM(p)]}, these factors are provided by the host cell and their expression depends on cell proliferation and differentiation (15). In the case of other viruses such as the adeno-associated viruses, the necessary functions can be supplied by helper viruses (5).

The genome of MVM(p) is organized in two overlapping transcription units controlled by two promoters, P4 and P38. P4 directs synthesis of the nonstructural proteins NS1 and NS2, while P38 regulates expression of the capsid proteins VP1 and VP2. Interest in parvoviruses stems from the striking specificity displayed by these viruses in their interactions with host cells and notably from the oncotropism of several of them. Parvoviruses can interfere with the appearance of spontaneous tumors and efficiently suppress experimental induction of oncogenesis (44). Furthermore, in vitro cell transformation increases the susceptibility of certain cells to killing by parvoviruses, suggesting that neoplastic transformation may trigger cell factors involved in cytotoxicity.

Increased sensitivity often correlates with increased viral DNA synthesis and gene expression (14, 45) but not necessarily with infectious virions production (21).

Despite several studies aimed at elucidating the contribution of each parvovirus determinant in cytotoxicity, much remains to be learned about how the viruses perturb their host cells and how transformation enhances cell sensitivity to parvoviruses. Recent results, however, suggest that NS1 is involved in parvovirus cytotoxicity, while NS2 is required for maximal cytopathic activity in human cell lines (7, 31, 32).

The direct involvement of NS proteins in cell killing was first shown in simian virus 40-transformed human cells, which had integrated into their genome the NS transcription unit, cloned under the control of an inducible promoter (long terminal repeat [LTR] of mouse mammary tumor virus) (9). NS accumulation in these clones produces a cytopathic effect within a few days of induction by dexamethasone (Dex). Yet resistant subclones have been isolated from these sensitive cell lines, suggesting that NS cytotoxicity may be modulated by cell factors. In normal rat fibroblasts containing the Dex-responsive LTR-NS construct, accumulation of NS1 product is not sufficient to kill the cells. Cytotoxicity is triggered only when the cells undergo certain metabolic changes dependent on oncogene expression (35). Furthermore, recent results, obtained with the same cell system, indicate that NS toxicity requires cell proliferation and correlates with alteration of cell cycle progression. NS1 expression induces an accumulation of sensitive cells in the G_2 phase (39), indicating that NS1 cytotoxicity involves cell factors associated with cell cycle regulation.

In addition to its role in the viral life cycle (such as DNA replication and regulation of the P38 and P4 promoters), NS1 can interfere negatively (18, 31, 43) or positively (49) with gene expression driven by certain heterologous promoters. Recent studies (30) have shown that NS1 displays transcription acti-

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vator-like properties. It can inhibit transcription, under conditions where it fails to bind a target promoter, by sequestering a factor or factors essential to the promoter's activity. Interestingly, the NS1 sequences responsible for transcriptional regulation and cytotoxicity are physically linked (30).

These various findings indicate that NS1 may alter the regulation of the synthesis and/or activity of essential cell proteins. Protein phosphorylation is the most frequent form of post-translational modification in eukaryotic cells, involved in controlling a multitude of cell proteins. It critically affects the physiological activity of several transcription factors (27), kinases, and phosphatases that regulate cell cycle progression. NS1 may interfere with the function(s) of one or more of these proteins, since its expression and toxicity correlate with a perturbation of the cell cycle (38, 39). This prompted us to examine the effects of NS1 on the synthesis and phosphorylation of cell proteins. Using a cell system where NS1 synthesis is inducible and two-dimensional electrophoresis, we have shown that NS1 interferes with phosphorylation of a 14-kDa polypeptide and with the synthesis of two proteins, a 50-kDa protein and a β-tubulin-like protein.

MATERIALS AND METHODS

Cell cultures. Rat fibroblasts were cultured in Dulbecco modified Eagle medium supplemented with 10 or 5% fetal calf serum. FRNS1-25 is a phenotypically normal cell line, stably transfected with a plasmid containing the NS1 coding sequence under the control of the LTR of mouse mammary tumor virus. FRNS1-25.EJ1 and FRNS1-25.MT4.1 are derivatives transformed with the c-Ha-*ras* and the polyomavirus middle T oncogenes, respectively (35).

NS1 gene expression was induced by incubating the cells with 10^{-5} M Dex (10^{-2} M stock solution in ethanol; Sigma).

Two-dimensional gel electrophoresis. (i) Radioactive labeling. Approximately 3×10^5 cells were plated on 60-mm-diameter plastic tissue culture dishes. After 1 day of culture, Dex was added to the culture medium for 9 h. Labeling was performed during the last 2 h of NS1 induction, by incubating the cells in a phosphate-free medium supplemented with ^{32}P-labeled orthophosphate (300 μCi/ml; Amersham).

(ii) Sample preparation. After labeling, the cells were rapidly washed with cold phosphate-buffered saline (PBS) and lysed on ice with 200 μl of lysis buffer (12 g of urea, 500 μl of Triton X-100, 500 mg of dithiothreitol [DTT], 500 μl of Pharmalyte 3-10, 50 μM NaF, 100 μM sodium orthovanadate, and 2 mM phenylmethylsulfonyl fluoride in 25 μl of distilled water). The lysates were immediately frozen in liquid nitrogen and stored at −80°C until used.

(iii) Gel electrophoresis. Using the Multiphor II system (Pharmacia), we subjected 60 μl of lysate, supplemented with 40 μl of sample buffer, to isoelectric focusing for 30,550 V · h in Immobiline DryStrips (pH 4 to 7, 11 cm; Pharmacia). Before use, the strips were rehydrated overnight in rehydration buffer (12 g of urea, 0.13 ml of Triton X-100, 0.13 ml of Pharmalyte 3-10, and 50 mg of DTT dissolved in 25 ml of distilled water). After isoelectric focusing, the gels were equilibrated twice for 15 min under gentle shaking, once in a solution containing 30 mg of DTT and 10 ml of equilibration buffer (20 ml of 0.5 M Tris-HCl [pH 6.8], 72 g of urea, 60 ml of glycerol, and 2 g of sodium dodecyl sulfate [SDS] adjusted to 200 ml with distilled water) and once in a solution containing 0.45 g of iodoacetamide dissolved in 10 ml of equilibration buffer. The proteins present in the equilibrated gels were then electrophoresed through the second-dimension gels of the type EXL Gel XL SDS gradient 12 to 14% (Pharmacia). In this case, runs were carried out at constant voltage (100 V) and at 20 mA for 45 min and 40 mA for 165 min. Some second-dimension runs were done through SDS–14% polyacrylamide gels under constant current. The gels were then dried, and the ^{32}P-labeled polypeptides were located by autoradiography at −80°C, using β-max hyperfilm (Amersham).

Amino acid microsequencing. Sample proteins were separated by two-dimensional electrophoresis and electroblotted onto polyvinylidene difluoride (PVDF) membranes as described by Matsudaira (34). Prior to in situ CNBr cleavage, the blot was stained with Coomassie blue. In situ CNBr protein cleavage was carried out as described by Stone et al. (46). The mixture of CNBr peptides was eluted from the membrane as described by Matsudaira (34). The eluted peptides were separated by SDS-PAGE and electroblotted onto PVDF membranes as described above. Alkylation of cysteine with acrylamide for sequencing was performed in situ (on the sequence) as described by Brune (8) and Carion et al. (11). Microsequencing of the electroblotted proteins was performed by automated Edman degradation of 1 to 10 pmol of protein in a Beckman LF 3400 protein microsequencer equipped with an on-line Gold 126 microgradient high-pressure liquid chromatography system and a model 168 Diode Array detector (Beckman Instruments, Inc., Fullerton, Calif.). All samples were sequenced by using the standard Beckman sequencer procedure 4. The phenylthiohydantoin amino acid derivative was quantitatively identified by reverse-phase high-pressure liquid chromatography on an ODS Spherogel micro phenylthiodydantoin column (3 by 2 by 150 mm; Beckman Instruments).

Detection of the NS1 protein. NS1 expression was visualized by immunoblotting. About 4×10^5 synchronized cells, treated or not with 10^{-5} M Dex for 9 h, were trypsinized and lysed in 2% SDS–50 mM Tris-HCl (pH 6.8)–10% glycerol–0.1% bromophenol blue–2% β-mercaptoethanol. After three cycles of freezing and boiling, samples were fractionated by SDS-PAGE (7.5% acrylamide) and electroblotted onto a nitrocellulose membrane in 20 mM Tris-HCl–150 mM glycine–20% methanol (pH 8). The blots were incubated with a polyclonal rabbit antiserum raised against a bacterial fusion protein containing a sequence characteristic of the carboxy-terminal region of the MVM(p) NS1 protein. Immunocomplexes were revealed with an alkaline phosphatase-linked anti-rabbit antibody (Protoblot kit; Promega).

Cell synchronization. Cells were synchronized in the G_1 phase with lovastatin, kindly provided by Merck Sharp & Dohme B.V., as previously described (28). Briefly, the cells were incubated for 24 h with 10 μM (final concentration) lovastatin as required for maximal cell accumulation in the G_1 phase. They were then released from the G_1 block by replacing the medium with fresh medium supplemented with 100 μM (final concentration) mevalonic acid. Dex (10^{-5} M [final concentration]) was added to the culture medium 9 h before the beginning of each phase. For two-dimensional gel electrophoresis, ^{32}P labeling was performed during the last 2 h of NS1 induction time.

Flow cytometry. We used flow cytometry to test the efficiency of synchronization and to monitor cell progression through the cell cycle. About 5×10^5 cells were trypsinized and washed with PBS. After centrifugation, the pellet was suspended in 1 ml of cold PBS and kept on ice for a few minutes. The cell suspension was then fixed by dropwise addition of 9 ml of precooled (−20°C) 80% ethanol, under violent shaking. Fixed samples were kept at −20 until used. For staining, the cells were centrifuged, resuspended in PBS, and treated with RNase A (100 μg/ml; Boehringer) and then with propidium iodide (50 μg/ml; Sigma) for 30 min at room temperature. Flow cytometry was carried out with a fluorescence-activated cell sorter (Becton Dickinson FACSscan).

Alkali treatment of ^{32}P-labeled proteins in polyacrylamide gels. Solubilized phosphoproteins were subjected to two-dimensional electrophoresis as described above. To prevent the loss of proteins during alkali treatment, the fixed gels were first soaked in 10% glutaraldehyde solution for 30 min at room temperature (glutaraldehyde promotes cross-linking of polypeptides within the gel). Next, the gels were treated or not with 1 M NaOH at 56°C for 1 h under gentle shaking. The alkali-treated gels were immersed in 10% acetic acid solution and washed for about 30 min with two changes of the acidic solution. The gels were then dried and autoradiographed with β-max hyperfilm.

RESULTS

Effect of NS1 expression on cell protein phosphorylation. To study the influence of NS1 expression on cell protein phosphorylation, we used cells in which the gene coding for the NS1 protein is conditionally expressed, i.e., cells with a stably integrated Dex-inducible LTR-NS1 construct. This is notably the case of strain FRNS1-25, derived from the established FR3T3 line of phenotypically normal rat fibroblasts. As recently reported (35), FRNS1-25 cells are resistant to NS1 expression, and addition of Dex does not significantly perturb their cycle. In contrast, derivatives transformed by the c-Ha-*ras* oncogene (FRNS1-25EJ1) or the polyomavirus middle T oncogene (FRNS1-25MT4-1) accumulate, after NS1 induction, in the S/G_2 phase of the cycle and die within a few days (35, 38, 39). We used two-dimensional gel electrophoresis to analyze the effect of NS1 expression on cell protein phosphorylation in these cell lines. Asynchronous cultures were treated for 0, 7, 18, 24, and 48 h with Dex, and ^{32}P labeling was performed for the last 2 h of the induction period. Figure 1 presents the two-dimensional gel electrophoresis patterns obtained from total phosphoproteins. In sensitive cells transformed by c-Ha-*ras* (FRNS1-25EJ1 [Fig. 1A and B]) or polyomavirus middle T (FRNS1-25MT4-1 [Fig. 1C and D]), NS1 expression appears to prevent phosphorylation of an acidic (pI ≈ 5) protein of about 14 kDa (p14). On autoradiographs obtained after ^{35}S labeling of FRNS1-25EJ1 cells, the intensity of the corresponding spot is only slightly lower after NS1 induction (data not shown), indicating that the drastic NS1-associated change affecting the p14 protein concerns its phosphorylation rather than its synthesis. This event occurs as early as 7 h after induction and is also observed after 18, 24, and 48 h of Dex

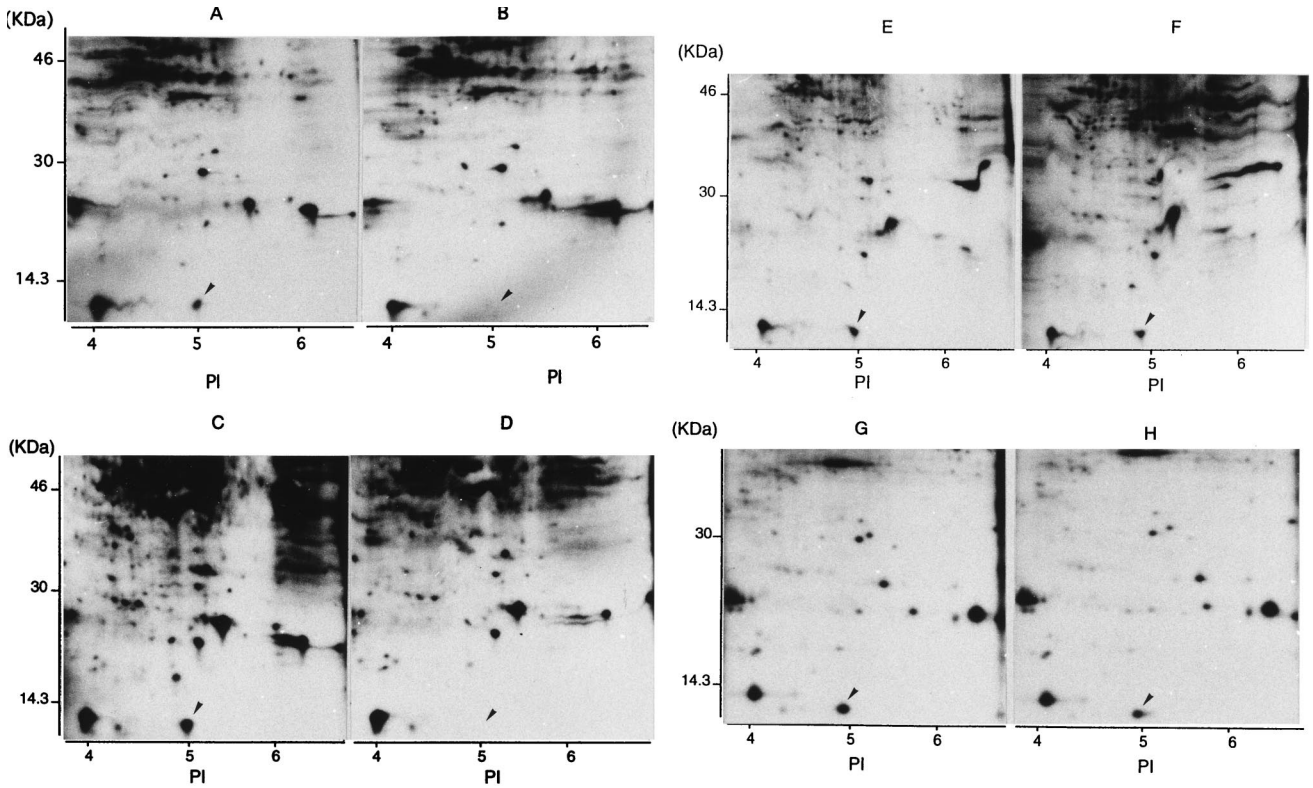


FIG. 1. Autoradiographs showing the effects of NS1 induction on cell protein phosphorylation. (A to D) Inducible NS1-sensitive rat fibroblasts transformed with the c-Ha-*ras* (FRNS1-25EJ1) (A and B) or polyomavirus middle T oncogene (FRNS1-25MT4-1) (C and D); (E and F) c-Ha-*ras*-transformed rat fibroblasts lacking the LTR-NS1 construct (FREJ4); (G and H) inducible NS1-resistant normal rat fibroblasts (FRNS1-25). As described in Materials and Methods, asynchronous cells were (B, D, F, and H) or were not (A, C, E, and G) incubated with 10^{-5} M Dex for 7 h and were ^{32}P-labeled for the last 2 h of the induction period. Total cell protein extract was subjected to two-dimensional electrophoresis. Second-dimension runs were done through gels of the type EXL Gel XL SDS gradient 12 to 14% (Pharmacia) (A to F) or through 14% polyacrylamide gels (G and H). Arrowheads indicate the protein (p14) whose phosphorylation pattern changes after NS1 induction.

treatment (data not shown). In the normal NS1-resistant parental cell line (FRNS1-25), p14 phosphorylation seems unaffected (Fig. 1E and F). Moreover, Dex treatment does not impair p14 phosphorylation in c-Ha-*ras*-transformed cells lacking the LTR-NS1 construct (FREJ4) (Fig. 1G and H), and so the effect must be mediated by NS1 rather than the inducer. The observed change in p14 phosphorylation is thus NS1 dependent and specific to NS1-sensitive cell lines.

Protein p14 is phosphorylated in early S phase, while NS1 can affect p14 phosphorylation at any time in the cell cycle. Since NS1 expression interferes with cell cycle progression by preventing mitosis (39), we examined the NS1 effect on p14 in relation to cell cycle progression. We synchronized NS1-sensitive c-Ha-*ras*-transformed cells (FRNS1-25EJ1) in the G_1 phase, using lovastatin and mevalonate as previously described (28). As depicted in Fig. 2, exponentially growing c-Ha-*ras*-transformed cells (FRNS1-25EJ1) were treated with lovastatin for 24 h. About 90% of the cells were shown by flow cytometry to be in the G_1 phase by the end of this period (Fig. 3D), a much higher proportion than among asynchronous control cells (Fig. 3C). To release the cells from the lovastatin-induced G_1 block, we incubated them with mevalonate. Ten hours postrelease, the cells synchronously began to enter the S phase (Fig. 3G). DNA synthesis peaked 14 to 15 h after addition of

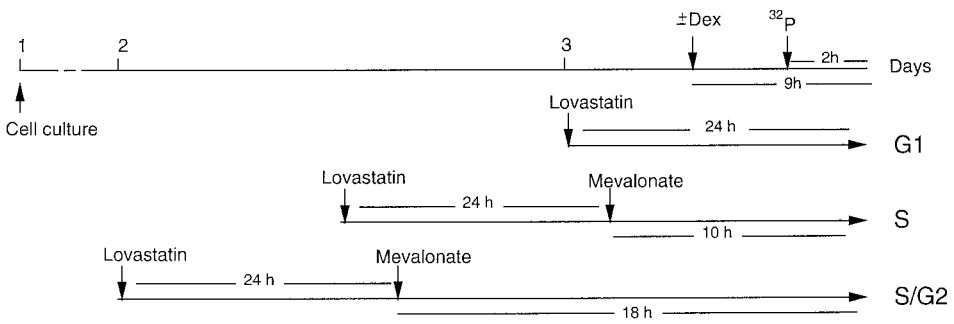
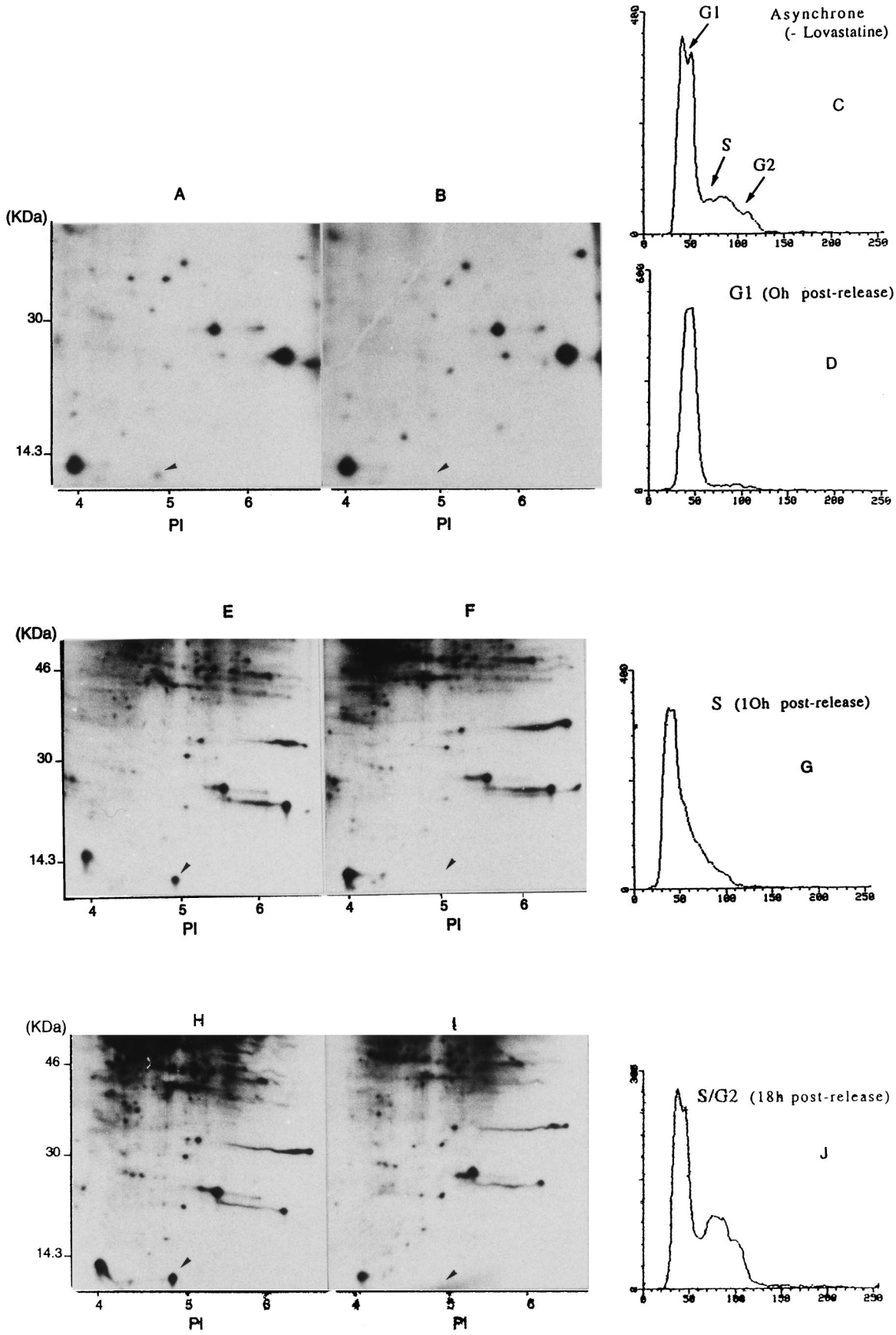


FIG. 2. Experimental procedure. Cells were synchronized in the G_1 phase by incubation in lovastatin for 24 h (see Materials and Methods). Cells were released from the G_1 block by incubation in fresh medium supplemented with mevalonate for 0 h (G_1), 10 h (S), or 18 h (S/G_2). For two-dimensional electrophoresis, the cells were incubated or not with 10^{-5} M Dex for 9 h and with [^{32}P]orthophosphate 2 h before total protein extraction.


A
B
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4
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G1
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D
E
F
(KDa)
46
30
14.3
S (1Oh post-release)
G
H
I
(KDa)
46
30
14.3
S/G2 (18h post-release)
J

FIG. 3. Effects of NS1 induction on p14 phosphorylation in the course of the cell cycle. The experimental procedure was as for Fig. 2. The inducible NS1-sensitive c-Ha-*ras*-transformed cell line (FRNS1-25EJ1) was G_1 synchronized with lovastatin (see Materials and Methods). ^{32}P-labeled proteins were extracted from cells treated with Dex for 9 h (B, F, and I) and from untreated cells (A, E, and H) at the following times after release from the G_1 block: 0 h (A and B), 10 h (E and F), and 18 h (H and I). Extracts were subjected to two-dimensional electrophoresis as described in Materials and Methods. Second-dimension electrophoresis was carried out in a gel of the type EXL Gel XL SDS gradient 12 to 14% (Pharmacia) (E, F, H, and I) or 14% polyacrylamide gels (A and B). Arrowheads indicates p14. The synchronization efficiency was tested by flow cytometry (see Materials and Methods). (C) Distribution of control cells (asynchronous cells) among the phases. (D, G, and J) Cells analyzed 0, 10, and 18 h, respectively, after cell release from the G_1 block.

mevalonate (data not shown), and the cells reached G_2 within 18 h (Fig. 3J). We tested the NS1 effect on p14 phosphorylation according to this timing, analyzing by two-dimensional gel electrophoresis the phosphorylation patterns of proteins extracted from cells in G_1 (Fig. 3A and B), S (Fig. 3E and F), and S/G_2 (Fig. 3H and I), with (Fig. 3B, F, and I) and without (Fig. 3A, E, and H) prior NS1 induction. Interestingly, p14 phosphorylation appears to change in the course of the cell cycle. The protein is slightly phosphorylated in G_1 (Fig. 3A) but much more so in S and S/G_2 (Fig. 3E and H, respectively). As shown above, NS1 prevents p14 phosphorylation whatever the phase (Fig. 3B, F, and I). Its action thus appears independent of the point in the cell cycle.

NS1 is phosphorylated during the S phase. We concurrently used Western blot analysis to monitor NS1 expression in the course of the cell cycle. Proteins extracted from induced synchronized cells were fractionated by SDS-PAGE (7.5% acrylamide). An antiserum raised against NS1 was used to visualize the separated phosphorylated and nonphosphorylated forms. The immunoblot shows (Fig. 4) that the phosphorylation status of NS1 changes during the cell cycle. In phases G_1 and S, only two bands, (83 and 84 kDa), corresponding to unphosphorylated and (slightly) phosphorylated NS1, appear. In the S/G_2 phase, the protein appears hyperphosphorylated. Since Dex was added to the culture medium 9 h before the cells entered each phase, we presume that hyperphosphorylation of NS1, observed at the beginning of the G_2 phase, occurs in the S rather than the G_2 phase of the cycle.

Alkali sensitivity of ^{32}P-labeled p14. Since phosphoryl groups linked to tyrosine (or threonine, to a lesser extent) are more resistant to alkali treatment than ones linked to serine residues, we tested the ability of the phosphorylated residues of p14 to resist alkali treatment. Incubation of polyacrylamide gels in alkali (1 M NaOH) is frequently used to detect phosphotyrosyl-containing cell proteins (6, 13), since such treatment yields labeled protein fractions enriched in phosphotyrosine (13, 20). A comparison of the autoradiographs of the alkali-treated gel (Fig. 5B) and untreated control (Fig. 5A) shows that the intensity of many spots remains unchanged after alkali treatment. The phosphoryl groups corresponding to these spots are thus essentially linked to tyrosine. In contrast, some spots appear very faint after alkali treatment, and so the phosphorylated residues of the corresponding proteins should essentially be serine and/or threonine. This is notably the case of p14.

Effect of NS1 expression on the synthesis of cell proteins. Since NS1 can regulate the expression of several parvovirus and nonparvovirus genes, we investigated how it affects the synthesis of cell proteins. Cell cultures were treated or not with 10^{-5} M Dex for 48 h. Total protein extracts of these cells were subjected to two-dimensional gel electrophoresis. Figure 6 shows the protein patterns obtained before and after Dex treatment, as revealed by Coomassie blue staining. Under our experimental conditions (pH range, 4 to 7), there appear in extracts of NS1-sensitive c-Ha-*ras*-transformed cells (FRNS1-25EJ1 [Fig. 6A and B]) two proteins whose accumulation seems altered as a result of NS1 induction: one of about 35 kDa (p35, pI $\approx$ 5) and one of about 50 kDa (p50, pI $\approx$ 6.5). In Dex-treated cells (Fig. 6B), p35 synthesis is inhibited, while p50 synthesis is stimulated. The same changes were observed 24 h of Dex treatment (data not shown). In the resistant (FRNS1-25 [Fig. 6E and F]) and NS1-free (FREJ4 [Fig. 6C and D]) cell lines, synthesis of these proteins remains unchanged after Dex treatment, indicating that the observed effects on p35 and p50 are linked to NS1 expression and cell sensitivity to NS1.

Protein blotting and microsequencing. We microsequenced p35 and p50 in an attempt to determine their nature. Total protein extract was electroblotted (see Materials and Methods) onto a PVDF membrane. After Coomassie blue staining, proteins of interest were cut from the membrane and directly microsequenced. In this way, we identified the N-terminal amino acid sequence of p35; the 25 N-terminal amino acids are MREIVHIQAGQCGNQIGPKFWEVIS, exactly matching the N terminus of β-tubulin. This tallies with the recent findings of Hughes and coworkers, who identified a β-tubulin with approximately the same pI and molecular weight (26).

We failed to obtain a sequence for p50 by direct N-terminal microsequencing, and so we performed in situ CNBr cleavage of this protein (see Materials and Methods). The eluted peptides were separated by SDS-PAGE and electroblotted onto PVDF membranes. We observed no peptides on the membrane, while identically treated bovine serum albumin (the positive reaction control) yielded four peptides on the PVDF membrane.

Due to the low level of p14 expression, we had to mix several

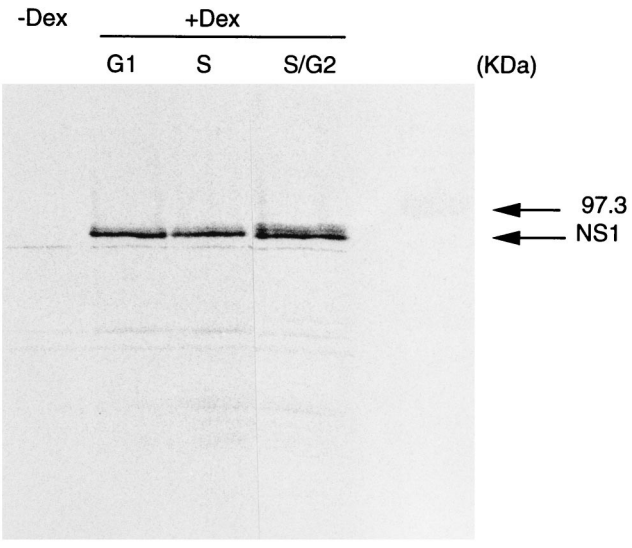

FIG. 4. Western blot analysis of NS1 according to the point in the cell cycle. The inducible c-Ha-*ras*-transformed, NS1-sensitive cell line (FRNSI-25EJ1) was synchronized with lovastatin (see Materials and Methods). NS1 synthesis was induced by incubation with 10^{-5} M Dex for 9 h. Proteins extracted from G_1-, S-, and S/G_2-phase cells were fractionated by SDS-PAGE (7.5% acrylamide) as described in Materials and Methods. -Dex indicates the control without NS1 induction.

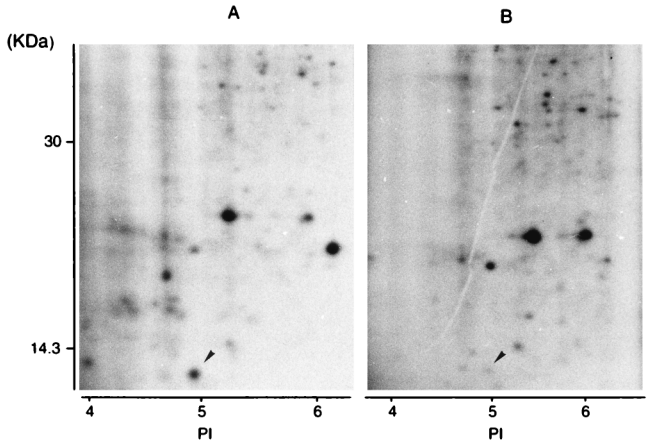

FIG. 5. Sensitivity of the p14 protein to alkali. ^{32}P-labeled proteins extracted from FRNS1-25EJ1 were subjected to two-dimensional electrophoresis (see Materials and Methods). The gels were then treated (B) or not (A) with 1 M NaOH for 1 h before fixing, drying, and autoradiography. Arrowheads indicate p14.

protein extracts, with either ^{32}P-labeled or unlabeled proteins. After electroblotting and staining, the membranes were autoradiographed and p14 was visualized by superimposing the autoradiograph on the membrane. N-terminal microsequencing of this peptide failed to reveal any sequence, probably because the amount of this protein is too low. Purification and concentration of this protein are in progress, to make sequencing possible.

DISCUSSION

The NS1 protein has been shown to possess several activities (endonuclease, helicase, ATPase, DNA binding, and activation of transcription) involved in various steps of the viral life cycle. NS1 is also the major effector of parvovirus cytopathogenicity. This has been shown by means of rat clones having stably integrated the Dex-responsive LTR-NS construct (35). NS proteins are reported to be toxic to transformed cells. NS1 alone kills as effectively as both NS proteins together (35). Yet in normal cells, it is innocuous. NS1 toxicity could result, at least in part, from a perturbation of the cell cycle, since the protein causes an arrest in S/G_2 in sensitive cell lines (38, 39). In this report, we demonstrate effects of NS1 on cell protein phosphorylation and expression in the inducible system described above.

NS1 interferes with phosphorylation of p14. Under the conditions used here (pH range, 4 to 7) to separate and detect phosphorylated proteins, NS1 reproducibly interferes with phosphorylation of protein p14, within 7 h of the addition of inducer. This early effect of NS1 is specific to NS1-sensitive transformed cells, since it is observed in FRNS1-25EJ1 and FRNS1-25MT4-1 cell lines which are transformed by oncogenes (c-Ha-*ras* and polyomavirus middle T, respectively) involved in different pathways of transformation. In the resistant normal parental cells (FRNS1-25), p14 is normally phosphorylated despite NS1 synthesis. The absence of p14 phosphorylation, after NS1 induction, thus correlates with oncogenic transformation and hence with NS1 toxicity. This confirms previous reports showing that cell factors, probably modulated by cell transformation, affect NS1 toxicity (35). Transformation of cultured cells is often accompanied by changes in cell growth, in turn related to changes in protein synthesis and phosphorylation (22). In this study, we also observed differences in protein phosphorylation between normal FRNS1-25 cells and their transformed derivatives (data not shown). The nature of the affected proteins and their roles in NS1 cytotoxicity remain to be determined.

Although NS1 is detected in inducible clones 2 to 6 h after addition of Dex to the culture medium, cell death occurs only after 3 to 5 days of treatment (9, 35). Hence, nonphosphorylation of p14 (after a 7-h induction of NS1) might be required for and even directly linked to cell killing via some disturbance of cell metabolism. Since NS1 interferes with cell cycle progression (38, 39), the disturbance may concern one or more cell cycle-regulating proteins. Phosphorylation is indeed the primary posttranslational regulatory mechanism controlling cell cycle progression, and many proteins undergo cell cycle-dependent changes in their levels of phosphorylation. Interestingly, our results obtained with synchronized cells suggest that p14 is phosphorylated during the S phase of the cycle. It is tempting to speculate that this protein might be directly or indirectly involved in the kinase cascade that leads to DNA replication. If so, the absence of p14 phosphorylation due to NS1 induction might hinder replication, causing S-phase arrest. In agreement with this possibility, NS1 has recently been reported to interfere with DNA synthesis (38): its expression in

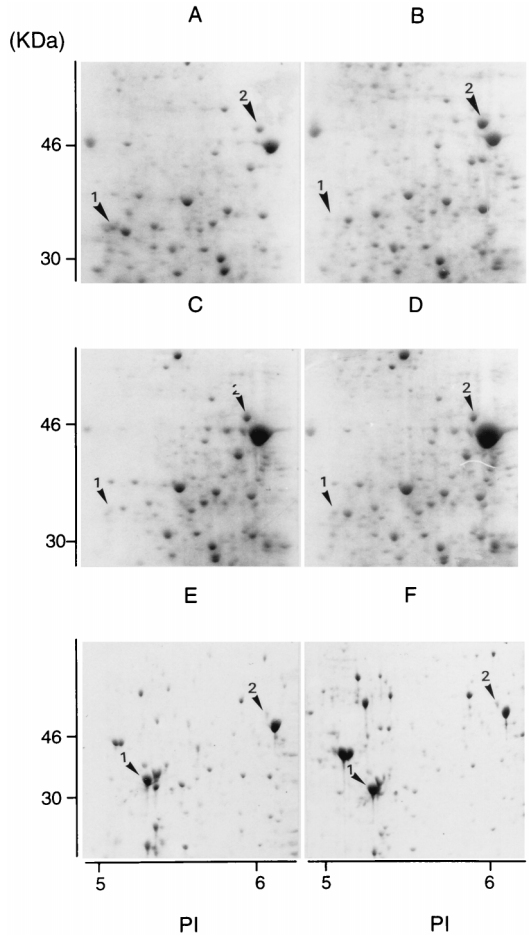

FIG. 6. Effect of NS1 on cell protein expression. Cells were (B, D, and F) or were not (A, C, and E) incubated with 10^{-5} M Dex. Protein extracts were subjected to two-dimensional electrophoresis (see Materials and Methods). (A and B) Inducible c-Ha-*ras*-transformed NS1-sensitive cells (FRNS1-25EJ1); (C and D) c-Ha-*ras*-transformed NS1-free control cells; (E and F) inducible normal NS1-resistant cells. Arrowheads indicate proteins whose expression pattern changes after NS1 induction.

sensitive cell lines correlates with a decreased capacity of the cells to incorporate bromodeoxyuridine within 15 h of NS1 induction. The adeno-associated virus replication protein (Rep78) has also been shown to inhibit cell DNA synthesis (50).

On the basis of the known properties of NS1, one of the following mechanisms might underlie the protein's ability to prevent p14 phosphorylation.

(i) Since NS1 can interfere with gene expression driven by certain heterologous promoters and also with the regulation of host cell gene expression (see below), it might directly or indirectly up- or down-regulate expression of an enzyme (a phosphatase or kinase, respectively) controlling the phosphorylation status of p14.

(ii) NS1 is able to interact with itself (37, 41) and with cell proteins (29). Indeed, it might disturb normal functions of phosphatase and kinase that control p14 phosphorylation by interacting with such enzymes. This possibility is supported by recent finding of Tung and coworkers, who reported that human immunodeficiency virus type 1 (HIV-1)-encoded protein complex NCp7-Vpr activates protein phosphatase 2A in vitro (48).

(iii) On the other hand, NS1 itself is a phosphoprotein. In vitro, it is phosphorylated by several kinases. This posttranslational modification seems to modulate some of the protein's activities, since dephosphorylation of NS1 correlates with a dramatic reduction of its in vitro ATPase, endonuclease, and helicase activities (36). In vitro, NS1 phosphorylation occurs exclusively on serine residues (40). Interestingly, the alkali stability test suggests that the same is true in p14. Furthermore, our immunoblotting data indicate that NS1 becomes hyperphosphorylated in the S/G_2 phase. Since both NS1 and p14 seem to be phosphorylated essentially on serine residues and during the same phase, they might be substrates of the same kinase. NS1 might compete with p14 for phosphorylation, thus interfering with p14 function.

NS1 interferes with the synthesis of certain cell proteins. Several studies have shown that the parvovirus nonstructural NS1 and Rep proteins can alter the regulation of host cell gene expression (24, 39) and regulate gene expression driven by parvovirus and nonparvovirus promoters (2–4, 16, 17, 23–25, 47). Such effects might contribute to parvovirus cytopathogenicity, a possibility supported by the presence, in parvovirus promoters, of several transcription factor-binding sequences (1, 19) required for transactivation by NS1. NS1 might thus deregulate expression of certain cell genes through interaction with cell proteins.

Under our experimental conditions (pH range, 4 to 7), NS1 reproducibly appears to alter the synthesis of two host cell proteins, β-tubulin (or a protein identical to β-tubulin at its N terminus) and an unidentified 50-kDa protein. The NS1-associated changes in p50 and β-tubulin accumulation might also be related to an effect of NS1 on their stability. But since this protein is known to up- and down-regulate heterologous promoters, we presume that these changes involve the expression rather than the stability of these proteins. This NS1 effect is observed only in NS1-sensitive cell lines, suggesting that it could be linked to NS1 toxicity. In eukaryotic cells, β-tubulin forms with α-tubulin a heterodimer which is a dynamic component of the cytoskeleton, involved in diverse processes including cell motility, determination of cell shape, and mitosis. It follows that altered regulation of β-tubulin synthesis might perturb these cell functions, possibly explaining why so few NS1-sensitive cells reach mitosis after NS1 induction. Cytotoxicity associated with decreased β-tubulin expression has been described in another system: in vitro, the MPP^+ neurotoxin (the oxidized form of *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) inhibits β-tubulin synthesis; the lack of this microtubule protein may underlie MPP^+ neurotoxicity (10).

The ability of the HIV-1 transactivator protein Tat to alter the synthesis of certain cell proteins may likewise be related to HIV-1 pathogenicity (42). Our findings further tally with a previous report showing that NS1 toxicity cosegregates with the protein's promoter-repressing ability (30). On the other hand, the NS1 protein of parvovirus H1 loses both its transactivating activity and its toxicity when its lysine 405 is mutated (33). All of these findings suggest that NS1 may interfere with the expression of vital cell genes. To date, only one cellular promoter has been tested in this regard: that of the gene coding for c-ErbA, the thyroid hormone receptor α. In cotransfection experiments, this promoter was shown to be activated by NS1 (49).

How the parvovirus NS1 product modulates expression of parvovirus and nonparvovirus promoters is presently unclear. The protein possesses, in its carboxy-terminal part responsible for toxicity, a transcription-activating domain (31). In cells transfected with a plasmid overexpressing NS1, the stimulation of responsive promoters decreases. This suggests that NS1 might sequester a factor or factors essential to promoter activity, a hypothesis supported by the ability of NS1 to interact with transcription factors such as SP1 (29). NS1 might also regulate gene expression by interacting directly with promoters. It was shown that this protein binds directly to the *tar* sequence of the MVM p38 promoter in a strictly ATP-dependent manner (12). Alternatively, NS1 might interfere indirectly with the activity of transcription factors by preventing their phosphorylation, since phosphorylation is important in vivo in the activation of transcription factors such as c-Jun, c-Myc, c-Myb, and CREB (13). We show here, furthermore, that NS1 impairs p14 phosphorylation. This might somehow perturb the phosphorylation cascade leading to transcription factor activation.

In conclusion, we provide evidence that the cytotoxicity of NS1 may involve its interference with p14 phosphorylation and with the synthesis of a β-tubulin-like polypeptide and a 50-kDa protein. We reported that the NS1 effect on p14 phosphorylation occurs very early during the induction period. This suggests strongly that the observed effect is directly linked to the mechanism implicated in cell damage. Since NS1 is multifunctional, several different pathways might be involved in these processes. Although the nature of p14 remains unknown, one might speculate that it plays a role in DNA replication, since p14 phosphorylation takes place during the S phase of the cell cycle. The low-level expression of this protein has prevented us from sequencing it. We are thus attempting its partial purification before sequencing. This should shed light on how NS1 induces cell death and hence on the mechanism of parvovirus oncosuppression.

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