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The NS1 Protein of the Autonomous Parvovirus Minute Virus of Mice Blocks Cellular DNA Replication: a Consequence of Lesions to the Chromatin?

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The nonstructural protein NS1 of the autonomous parvovirus minute virus of mice interferes with cell division and can cause cell death, depending on the cell transformation state. Upon infection, the synthesis of NS1 protein is massively initiated during S phase. In this article, we show that minute virus of mice-infected cells accumulate in this phase. To investigate the link between NS1 accumulation and S-phase arrest, we have used stably transfected cells in which NS1 expression is under the control of a glucocorticoid-inducible promoter (the long terminal repeat of mouse mammary tumor virus). NS1 expression interferes with cell DNA replication, and consequently, the cell cycle stops in S phase. NS1 expression also induces nicks in the cell chromatin, as detected by an in situ nick translation assay. The nicks are observed several hours before any cell cycle perturbation. As cell cycle arrest is a common consequence of DNA damage, we propose that NS1 exerts its cytostatic activity by inducing lesions in cell chromatin.

Autonomous parvoviruses are single-stranded DNA viruses that infect a wide range of hosts, including humans. Their low genomic complexity (size, about 5 kb) makes them highly dependent on the host cell to accomplish their viral cycle. Special proliferation and differentiation states are thus required to make cells permissive to parvovirus infection (for a review, see reference 6).

Particularly, early events in the viral cycle depend on cell factors associated with the S phase of the cell cycle. One such event is the conversion of the incoming single-stranded viral DNA into a double-stranded replicative form, an early step in parvovirus genome amplification and expression (20). Unlike oncogenic viruses, parvoviruses are unable to stimulate cell proliferation and consequently depend on the proliferation state of the cells.

The parvovirus cycle is also highly dependent on the nonstructural proteins (NS), which regulate the viral cycle at different levels. First, the nonstructural protein NS1 of the autonomous parvovirus minute virus of mice (MVMp) regulates expression of the parvovirus genes through activation of the parvovirus promoters P4 and P38. Second, during viral replication, NS1 exerts DNA binding, endonuclease, and helicase activities on the parvoviral genome. Probably because of this pleiotropic activity, NS1 synthesis is an early event in the viral life cycle that occurs as infected cells enter the S phase (6).

NS1 protein expression is also lethal in transformed cell lines. This cytotoxic property of NS1 was demonstrated by a study of stably transfected cell lines in which expression of NS1 is under the control of a glucocorticoid-inducible promoter, the long terminal repeat (LTR) of mouse mammary tumor virus (MMTV) (3, 16). Depending on the cell line, cell death occurs after an NS1 expression period of 3 to 5 days, during which cell growth is delayed or completely blocked. This cytostatic potential correlates with accumulation of the NS1-expressing cells in the G_2 phase (17). The molecular basis of NS1 cytostatic activity and the components involved remain unknown.

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As thymidine incorporation is massively reduced in MVMp-infected cells (6, 11), we have investigated how MVMp infection affects the cell cycle. Because of the cytostatic action of NS1, we investigated the role of the NS1 protein in inhibiting cell DNA replication. Insights into this role were gained through the use of stably transfected cell lines with inducible NS1 expression. Since cell cycle arrest is a common consequence of DNA damage and since NS1 displays endonucleolytic activity, we investigated whether NS1 might exert its cytostatic action by inducing nicks in the cell chromatin. This hypothesis was approached by looking for nicks in the chromatin of NS1-expressing cells.

MATERIALS AND METHODS

Cells and viruses. The simian virus 40 (SV40)-transformed newborn human kidney cell line NBE is cultivated in minimal essential medium supplemented with 5% fetal calf serum (FCS) (19).

The FRNS125 line and its derivatives transformed with the c-Ha-*ras* (FRNS125EJ1) or polyomavirus middle T (FRNS125MT4-1) oncogene are rat fibroblasts stably transfected with an MMTV LTR-directed molecular clone of the NS1 coding sequence of MVMp (16). FREJ4 cells are rat fibroblasts transformed by the c-Ha-*ras* oncogene (22). These lines are cultivated in Dulbecco's modified essential medium supplemented with 10% FCS and 1% sodium pyruvate. MVMp infection is carried out as follows. The cells are rinsed with phosphate-buffered saline (PBS) (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 140 mM NaCl, 2.7 mM KCl [pH 7.2 to 7.4]) supplemented with $CaCl_2$ (0.9 mM) and $MgSO_4$ (0.9 mM) (PBS-Ca-Mg). The cells are then incubated for 1 h in 1 ml of PBS-Ca-Mg buffer with or without the virus (multiplicity of infection, 10 infectious particles per cell). The cells are harvested after another incubation in culture medium at 37°C.

Synchronization in G_1. Cells are incubated for 24 h in the presence of lovastatin (10 mM), kindly provided by Merck Sharp & Dohme B.V., and prepared as described by Keyomarsi et al. (14). The inducer dexamethasone (Dex) (10^{-5} M) (Sigma Chemical Co.) is added or not to the culture when the G_1-blocked cells are released by replacing the blocking medium with fresh medium containing 1 mM mevalonate (Sigma Chemical Co).

Bromodeoxyuridine (BrdU) labeling. After a 30-min culture period in the presence of 100 μM BrdU (Sigma Chemical Co.), the cells are trypsinized, rinsed with PBS, suspended in 1 ml of PBS, and cooled on ice for 5 min. Cell suspensions are then fixed by dropwise addition of 9 ml of precooled (−20°C) 80% ethanol under violent shaking. Fixed samples are kept at −20°C until used.

Fixed cells are rinsed with PBS, and 10^6 to 10^7 cells are resuspended in 175 μl of PBS and added to 2 N HCl (700 μl) containing 0.2 mg of pepsin per ml. Digestion in the dark at 37°C is stopped after 30 min by neutralization with 1.875 ml of 1 M Tris base (pH 11). The cells are then rinsed with PBS and suspended in 50 μl of PBS–10% FCS containing 5 mg of anti-BrdU antibody coupled to

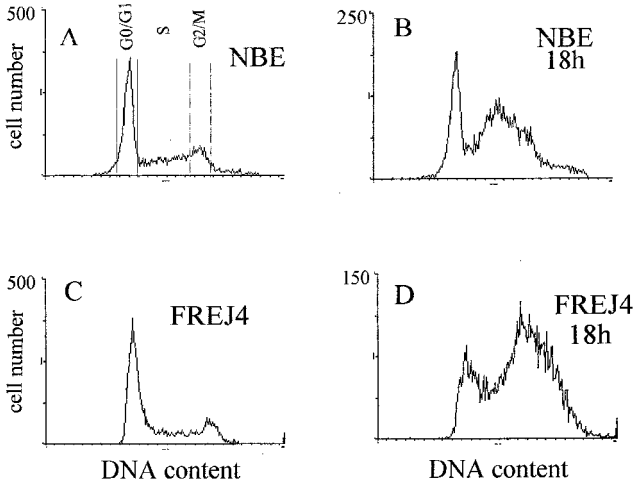

FIG. 1. Effect of MVMp infection on cell cycle distribution of human NBE (A and B) and rat fibroblast (C and D) cell lines. Exponentially growing cultures were infected with MVMp (multiplicity of infection, 10) (B and D) or were mock infected (A and C). Cells were harvested after an 18-h incubation and processed for flow cytometric analysis. The distribution of 10,000 cells is shown as a function of PI fluorescence, making it possible to distinguish the G_0/G_1, S, and G_2/M components as indicated in panel A.

fluorescein isothiocyanate (FITC) per ml. After a 1-h incubation on ice in the dark, the cells are rinsed with PBS and resuspended in 500 μl of PBS containing propidium iodide (PI) (20 mg/ml) (Sigma Chemical Co.) and RNase A (1 mg/ml) (Boehringer Mannheim). After a 20-min incubation, cell-associated fluorescence (both PI and FITC) is measured by flow cytometry (FACS; Becton Dickinson) using the Lysis II program (Becton Dickinson).

Nick detection in cell chromatin. Nicks are detected by the in situ nick translation assay described by Yang et al. (23). Briefly, cells cultivated on coverslips are rinsed with PBS and fixed by incubation for 5 min on ice in a freshly prepared 1% solution of paraformaldehyde in PBS. The cells are then rinsed with cold PBS and fixed in a precooled (−20°C) 80% ethanol solution for 15 min on ice. After another rinse with cold PBS, the cells are incubated for 15 min on ice in a cold solution of 0.1% Triton X-100 in PBS and again rinsed with cold PBS. Nucleotide incorporation is then performed in the dark for 1 h at 37°C by incubating cells in 100 μl of nick translation buffer (50 mM Tris-HCl [pH 7.5], 1mM dithiothreitol, 10 mM $MgCl_2$) containing 10 U of DNA polymerase I, dATP, dCTP, and dGTP (each at 1.4 μM) and dUTP-FITC (4 μM) (all from Boehringer Mannheim). The cells are then rinsed with a solution containing 50 mM Tris (pH 7.5) and 1 mM EDTA and then with PBS. The fluorescence signal is further amplified in two steps: first, a 2-h incubation in the dark at 37°C in hybridoma supernatant directed against FITC (kindly provided by J.-D. Franssen) and second, after rinsing with PBS, a 2-h incubation at 37°C in the dark with FITC-coupled goat anti-mouse antibodies (1:50 dilution in PBS–10% FCS) (Sigma Chemical Co.). After a last rinsing with PBS, coverslips are mounted and the cells are analyzed by fluorescence microscopy.

Pictures were taken with an MTI CCD72 video camera (integrated for 5 s). The image was visualized on the computer screen, enabling us to quantify the nuclear fluorescence with the help of a National Institutes of Health image program (public domain).

RESULTS

MVMp-infected cells accumulate in the S phase. To study how parvovirus infection interferes with cell division, we ob-

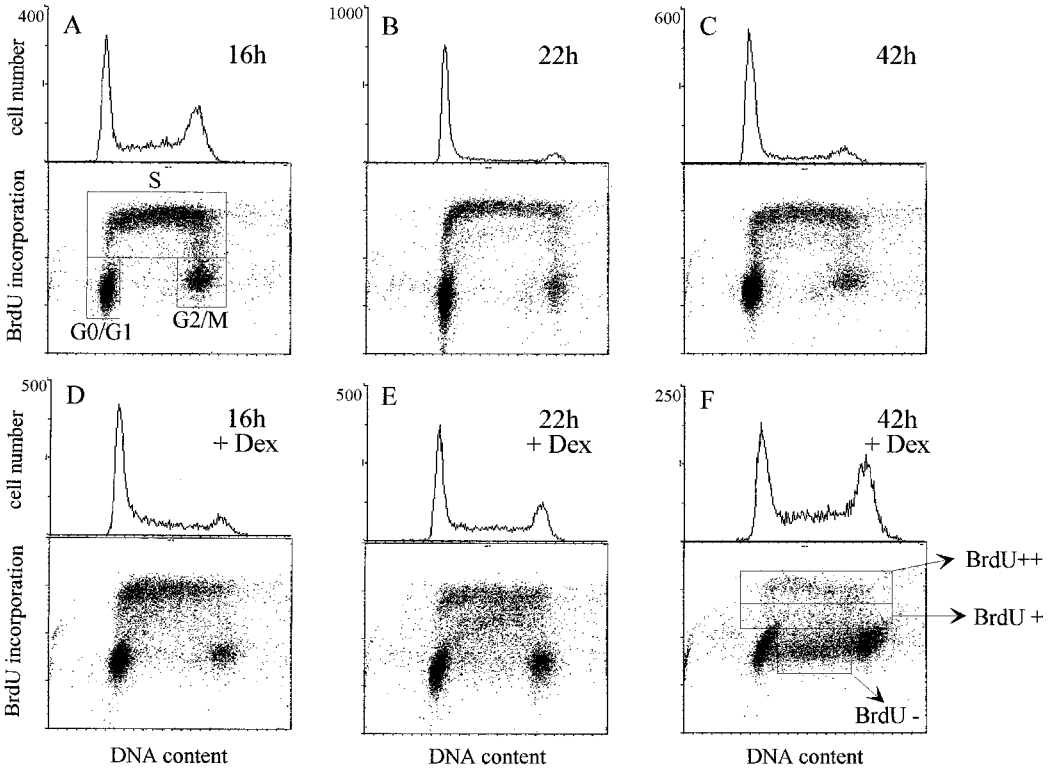

FIG. 2. Effect of NS1 expression on cell DNA synthesis in inducible transfectant rat fibroblasts transformed by the c-Ha-*ras* oncogene (FRNS125EJ1 cells). Cells were synchronized by incubation for 24 h in the presence of lovastatin. The G_1-blocked cells were then released by replacing the blocking medium with fresh medium containing mevalonate and the inducer Dex (D, E, and F) or no inducer (A, B, and C). At different times after the release (A and D, 16 h; B and E, 22 h; C and F, 42 h), the cells were cultured for 30 min in the presence of BrdU before being harvested and processed for flow cytometry. Lower panels show the level of BrdU incorporation (green fluorescence) of 10,000 cells versus their DNA content (red fluorescence). Upper panels show the corresponding cell distribution as a function of DNA content (as described in Fig. 1). Cells in G_0/G_1, S, or G_2/M phase are distinguished as presented in dot plot A. Cells displaying a high (BrdU++), medium (BrdU+), or low (BrdU−) level of BrdU incorporation are distinguished as in dot plot F.

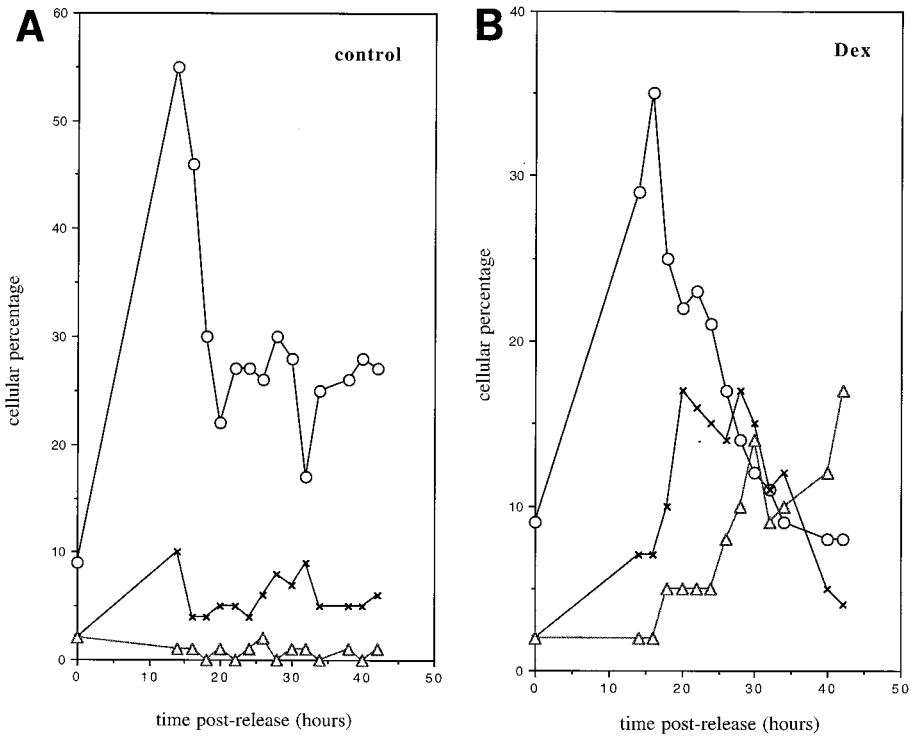

FIG. 3. Kinetics of NS1 interference with cell DNA synthesis in synchronized inducible transfectant rat fibroblasts transformed by the c-Ha-*ras* oncogene (FRNS125EJ1). The cells were synchronized by incubation for 24 h in the presence of lovastatin. The G_1-blocked cells were then released by replacing the blocking medium with fresh medium containing mevalonate and the inducer Dex (B) or no inducer (A). At different times after the release, cells were cultured for 30 min in the presence of BrdU before being harvested and processed for flow cytometry. Percentages of cells incorporating high levels (BrdU++) (○), medium levels (BrdU+) (x), or low levels (BrdU−) (△) of BrdU are distinguished as shown in dot plot F of Fig. 2.

served the consequences of MVMp infection on cell distribution in the different phases of the cell cycle. This was done by using a human newborn kidney cell line transformed by SV40 (NBE) and rat fibroblasts transformed by the Ha-*ras* oncogene (FREJ4) and by using flow cytometry, which makes it possible to distinguish cells in the G_0/G_1, S, and G_2/M phases on the basis of their DNA content: single, intermediate, and double, respectively. A suspension of fixed cells was stained with the intercalating dye PI, which associates a red fluorescence with each cell, proportional to its DNA content. The red fluorescence distribution of mock-treated and MVMp-infected cells is presented in Fig. 1. This figure indicates that after 18 h, MVMp-infected cells are massively accumulated in the S phase. This perturbed distribution is maintained several hours later, suggesting an arrest of the infected cells in the S phase.

NS1 prevents replication of cell DNA. To investigate the role of NS1 in infection-triggered S-phase accumulation, we examined whether NS1 expression interferes with cell DNA replication. We used rat fibroblast cell lines stably transfected with the coding sequence of NS1 under the control of a glucocorticoid-inducible promoter, LTR of MMTV. When NS1 expression is triggered by the inducer Dex, transformed rat fibroblasts stop growing and die after 2 to 3 days. We studied the consequences of NS1 expression on the cell cycle in a synchronized culture of rat fibroblasts that displayed inducible NS1 expression and had been transformed by the Ha-*ras* oncogene (the FRNS125EJ1 cell line). Cell cycle phases were distinguished by flow cytometry, and two parameters were measured: (i) cell DNA content (measured by PI staining) and (ii) the level of cell DNA synthesis (measured by BrdU incorporation).

Cells were first synchronized in G_1 phase by incubation in the presence of lovastatin, an inhibitor of 3-hydroxyl-3-methylglutaryl-coenzyme A reductase that catalyzes the synthesis of mevalonate (12, 14). After 24 h, the cells were released from the G_1 block by incubation in lovastatin-free, mevalonate-containing medium. At that time, Dex was added to induce NS1 expression, and cells were harvested every 2 h in both induced and control cultures after a 30-min incubation in the presence of BrdU. Cells replicating their DNA during that interval incorporate BrdU into their chromatin. Harvested cells were fixed and their BrdU incorporation levels and DNA contents were measured as described in Materials and Methods. The presence of BrdU was detected with an antibody coupled to the green-fluorescing molecule FITC, and the DNA content was determined with the red-fluorescing intercalating dye PI. Both fluorescences were measured in samples of 10,000 cells by flow cytometry. The results are displayed in Fig. 2. The G_0/G_1- and G_2/M-phase cells have a basal nonspecific green fluorescence, corresponding to no BrdU incorporation, and display a single or double DNA content, respectively. S-phase cells show an intermediate red fluorescence (and thus an intermediate DNA content) and a high green fluorescence corresponding to a high level of BrdU incorporation (Fig. 2A).

Kinetic studies showed that both Dex-treated and control cultures first incorporate BrdU at a high rate (16 h postrelease; Fig. 2A and D). A few hours later (22 h), the NS1-expressing S-phase cells incorporate BrdU less efficiently than control cells (22 h postrelease; Fig. 2B and E). Finally, Dex-treated cells with an intermediate DNA content cease to incorporate BrdU, indicating an arrest of the cells in S phase (42 h postrelease; Fig. 2C and F). On the basis of these diagrams, gates

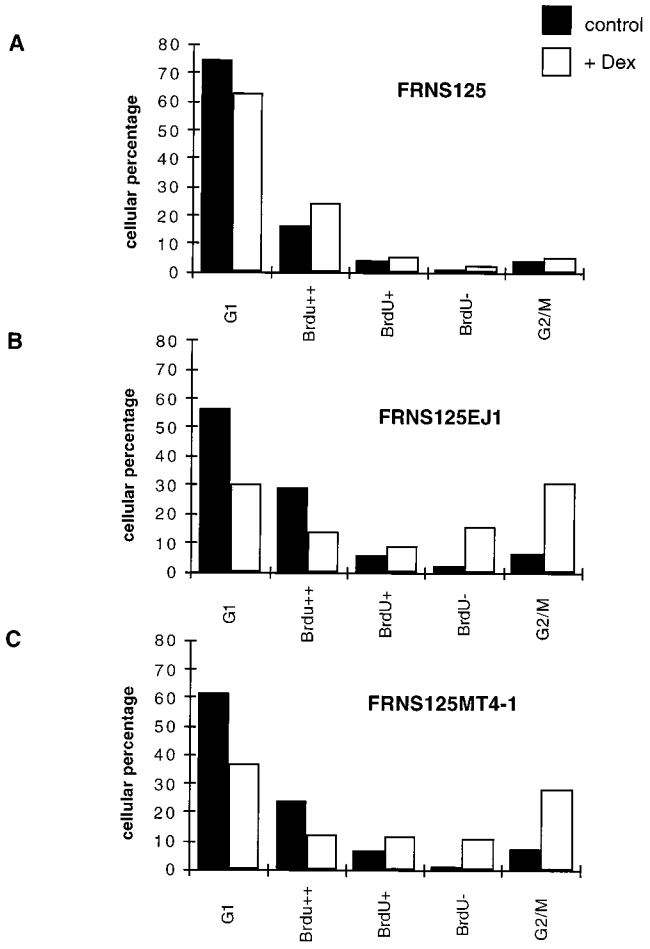

FIG. 4. Quantification of NS1 interference with cell cycle distribution and DNA synthesis in nontransformed cells (FRNS125) (A) and in inducible transfectant rat fibroblasts transformed either by the c-Ha-*ras* oncogene (FRNS125EJ1) (B) or by the middle T oncogene of polyomavirus (FRNS125MT4-1) (C). Exponentially growing (black) and 24-h-Dex-treated (white) cultures were incubated for 30 min in the presence of BrdU before being harvested and analyzed by flow cytometry for both BrdU incorporation and DNA content. Cell percentages in G_0/G_1, BrdU++, BrdU+, BrdU−, and G_2/M were calculated as shown in Fig. 2A and F.

were defined to distinguish cells with an intermediate DNA content according to the rate of BrdU incorporation and thus DNA replication (Fig. 2F). Three gates were defined to distinguish cells with high, intermediate, and background green fluorescence (BrdU++, BrdU+, and BrdU−, respectively), and the percentages of cells in these three regions were calculated and plotted according to time postrelease (Fig. 3). NS1 expression clearly interferes with cell DNA replication, since BdrU incorporation first slows down and then ceases completely in cells expressing NS1.

We then examined whether NS1 expression could trigger S-phase arrest in other stable transfectants displaying inducible expression of the parvovirus protein (Fig. 4). BrdU incorporation was measured in three cell lines: a normal rat fibroblast line (FRNS125) and two derivatives thereof, one (FRNS125EJ1) transformed by the Ha-*ras* oncogene and one (FRNS125MT4-1) transformed by the polyomavirus middle T oncogene. The distributions of cells among the five gates described in Fig. 2A (for G_1 and G_2/M cells) and Fig. 2F (for BrdU++, BrdU+, and BrdU− cells) in untreated and Dex-treated cultures were compared (Fig. 4). Once NS1 is expressed, both transformed cell lines stop growing (16) and the S-phase cells no longer incorporate BrdU (Fig. 4B and C). In contrast, NS1 expression does not modify BrdU incorporation by nontransformed cells (Fig. 4A). The NS1 protein thus blocks cell DNA replication in transformed cell lines, which are sensitive to the protein cytotoxicity, but not in nontransformed cells, which prove resistant to NS1.

NS1 induces nicks in the cell chromatin. Since cell cycle arrest is a common consequence of chromatin damage, we investigated whether NS1 might exert its cytostatic activity by inducing nicks in cell DNA. To this end, we sought to detect free 3′ OH ends in the chromatin of NS1-expressing transformed rat fibroblasts (FRNS125EJ1). The chromatin of cells fixed on coverslips was subjected to an in situ nick translation assay. In this in vitro assay, DNA polymerase I incorporates a fluorescently labeled base analog (FITC-dUTP) at nicking sites, associating a green fluorescence with the nuclei. The signal is then amplified as described in Materials and Methods. The fluorescence micrographs presented in Fig. 5A were obtained with synchronized transformed rat fibroblasts 14 h (Fig. 5A1) and 48 h (Fig. 5A2 and 5A3) after G_1 block release. A comparison of control (Fig. 5A2) and NS1-expressing (Fig. 5A1 and 5A3) cells reveals that NS1 expression induces higher nuclear labeling, indicative of numerous free 3′ OH ends. The nuclear fluorescence detected in control cells (Fig. 5A2) is not due to signal amplification but reflects the presence of a background level of free 3′ OH in cells fixed and stained by this treatment.

Furthermore, the nuclei of NS1-expressing cells appear larger than those of control cells. This probably reflects a tendency of these nuclei to spread out rather than an actual increase in cell size, as indicated by flow cytometry cell size measurements and phase-contrast microscopy (data not shown). Such a morphological change in other cells displaying arrested proliferation, e.g., cells expressing human immunodeficiency virus type 1 Vpr, has already been reported (18).

The nuclear fluorescence of Dex-treated and control cells was quantified 14 h after G_1 block release by analyzing digitalized video camera pictures (see Materials and Methods) of synchronized transformed rat fibroblasts (300 cells from a representative experiment) (Fig. 5B). NS1-expressing cells (Fig. 5B2) fluoresce more intensely than untreated cells (Fig. 5B1). Dex treatment does not induce any labeling or spreading of the nuclei of either control cells lacking the LTR-NS insert (FREJ4) or NS-1-resistant nontransformed rat fibroblasts (FRNS125) (data not shown). To make sure that the labeling observed in NS1-expressing cells is not merely an artifact due to increased accessibility of cell chromatin when NS1 is expressed, we examined cell chromatin accessibility to DNAse I (moderate treatment was applied before the base analog incorporation step). The nuclear fluorescence of NS1-expressing cells proved no greater than that of control cells (data not shown), so the increased labeling observed in NS1-expressing cells in the experiment without DNAse I most probably reflects NS1-induced nick generation in the cell chromatin. We detected the indicative fluorescence as early as 14 h after G_1 block release (Fig. 5A1 and 5B), i.e., several hours before any detectable cell cycle perturbation.

DISCUSSION

We here show that MVMp infection causes the accumulation of cells in the S phase. Most infected cells have a higher DNA content than cells in G_1, so MVMp infection must allow entry into the S phase and initiation of replication. Yet because

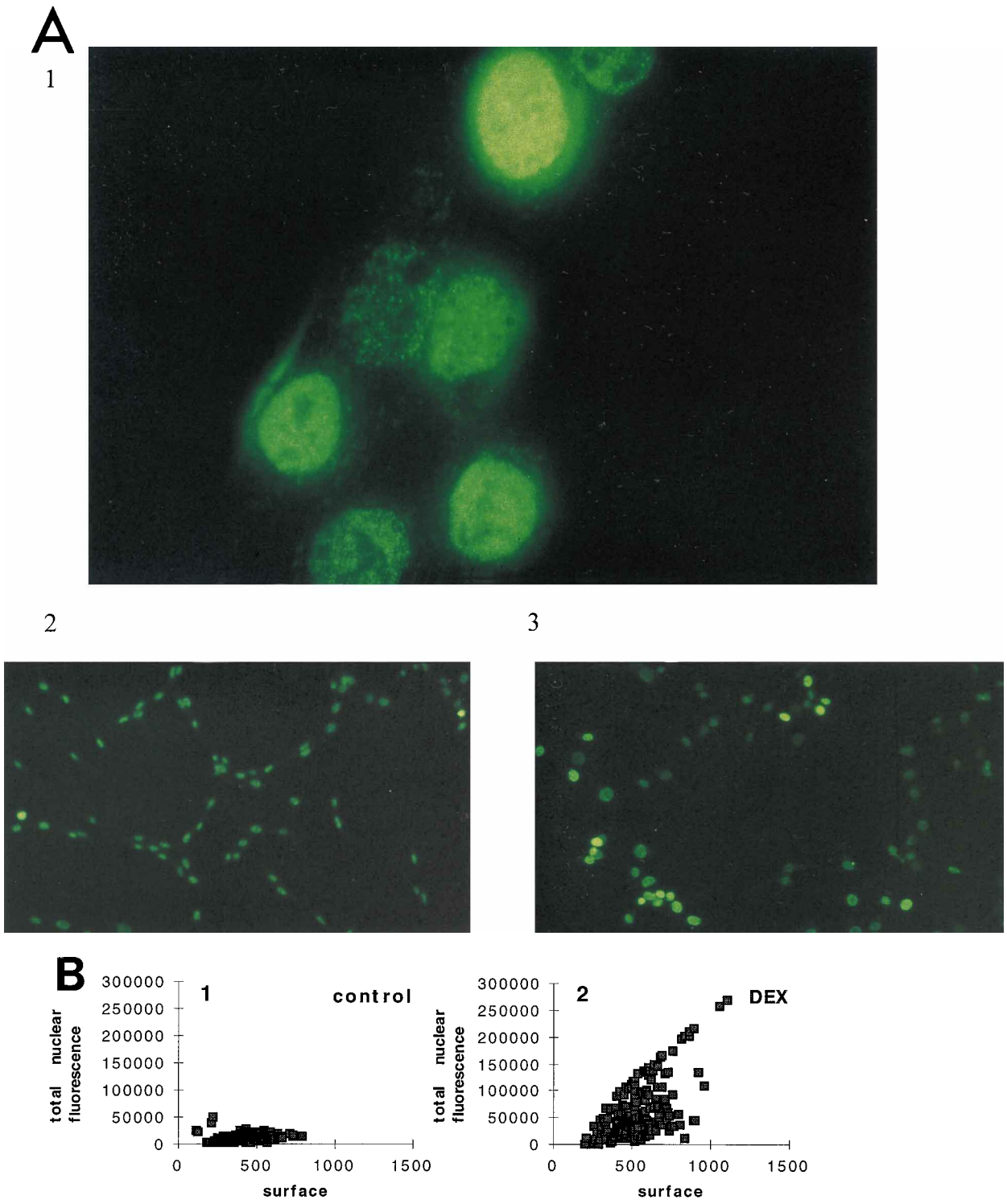

FIG. 5. Effect of NS1 on the presence of nicks in the cell chromatin of inducible transfectant rat fibroblasts transformed by the c-Ha-*ras* oncogene (FRNS125EJ1). Fluorescence micrographs of nuclear labeling in control cells (A2) and in NS1-expressing cells (A1 and A3) after the in situ nick translation (ISNT) assay are shown. Micrographs A2 and A3 were taken at the same magnification. Panel B shows quantification of nuclear fluorescence in NS1-expressing (B2) and control (B1) cells. ISNT assays were performed on lovastatin-synchronized cells, 14 h (A1 and B) and 48 h (A2 and A3) after G_1 block release, with Dex added to the culture medium at time zero (A1, A3, and B2) or not (A2 and B1).

of MVMp amplification, viral DNA probably also increases the total DNA content of infected cells, so we cannot judge how early or late in the S phase the arrest occurs. Our observations are in good agreement with previous ones showing that autonomous parvovirus infection requires S-phase-associated cell factors (20) and that MVMp infection causes a decrease in thymidine incorporation into cell DNA (11) but allows initiation of cell DNA replication (6). This interference occurs early

in infection, when there is de novo synthesis of viral proteins but little synthesis of viral DNA. This suggests that the mechanism involves viral proteins rather than titration of cell replication factors by viral DNA (6).

We here show that the NS1 protein is indeed involved, since cell DNA replication slows down and stops when NS1 is expressed upon induction in stably transfected cell lines. This was demonstrated by measuring BrdU incorporation, which progressively comes to a halt. We cannot exclude decreased BrdU uptake as an explanation of this observation, but the fact that BrdU incorporation and cell growth cease simultaneously makes this possibility seem unlikely. BrdU incorporation stops only in transformed cell lines, which stop growing when they express NS1, and not in NS1-resistant nontransformed rat fibroblasts, whose growth rate is only slightly reduced (16).

The cytostatic potential of NS1 has already been reported (3, 16), as has its ability to perturb the cell cycle (17). Other viral proteins, e.g., Vpr of human immunodeficiency virus (9), E1 of bovine papillomavirus (2), and Rep of adeno-associated virus (24, 25), are reported to block cell proliferation. The underlying mechanisms remain elusive. As a transcription regulator, NS1 might exert its cytostatic activity by regulating the expression of cell factors involved in cell cycle control. An alternative, but not exclusive, hypothesis is that as an endonuclease, NS1 might cause DNA damage, an event known to interfere with cell proliferation (reviewed in reference 13). Our results support this second hypothesis, since nicks are indeed detected in the chromatin of NS1-expressing cells and since these DNA lesions appear before any detectable perturbation of the cell cycle. Accordingly, NS1 can inhibit heterologous replicons (SV40-MVMp hybrids or Epstein-Barr virus) (21); this suggests that it might also interact with targets resembling parvovirus sequences in the cell DNA. To ensure parvovirus DNA replication, NS1 exerts its endonuclease activity at two sites, one at each end of the viral genome (reviewed in reference 7). NS1 is presumed to bind to the sequence ACCA (5) and to introduce a nick at a specific site within the nick consensus sequence CTWWTCA. The occurrence of both sequences is very frequent in the mammalian genome, but the presence of both sites close together is not sufficient to stimulate the endonuclease activity of NS1. The precise location of a bipartite site, allowing the binding of a parvovirus initiation factor, is essential for promoting NS1 nicking of the right-end sequence (4). Nevertheless, it is very likely that sequences allowing NS1 endonuclease activity exist in the cell chromatin.

Whether the NS1-induced nicks observed in the cell genome are produced by the NS1 endonuclease remains to be ascertained. This could be done by demonstrating covalent binding of NS1 to cell DNA nick sites. Alternatively, NS1 might activate a cell endonuclease in NS1-sensitive cells, as observed during apoptosis. In our system, we detected no signs of apoptosis, such as chromatin condensation or DNA ladders, in cells killed by NS1 (data not shown). It should be stressed that the nuclear labeling observed in NS1-expressing cells after our in situ nick translation assay (Fig. 5A) is very different from that obtained when apoptosis occurs (10), as observed, e.g., in cells expressing the adenovirus E1A protein (26). First, the fluorescent labeling is less intense than that in apoptotic nuclei, and its quantification requires signal amplification, which is not necessary to detect apoptosis. This is indicative of fewer nicks per nucleus. Second, no chromatin condensation or fragmentation is observed, even at the latest times after NS1 induction tested. These differences suggest that the nicks induced by NS1 in cell chromatin are unrelated to apoptosis.

NS1-triggered early nicking of cell DNA is observed shortly after NS1-induced dephosphorylation of protein p14, which is correlated with NS1 sensitivity (1). Whether this phenomenon is related to the DNA lesions and/or to inhibition of cell DNA synthesis remains to be determined.

Also striking is the heterogeneity of nuclear labeling among cells of a same NS1-expressing clone, reflecting considerable variability in the number of nicks from one cell to the other. An explanation might lie in the fact that the different cells variously express the protein, as detected in in situ immunofluorescence experiments (data not shown). Such heterogeneity in the expression of a cloned gene has been reported for the β-galactosidase gene under the control of the Dex-inducible promoter LTR of MMTV (15).

Variable NS1 expression might also explain why NS1-expressing clones and MVMp-infected cells show a different distribution of cells among the cell cycle phases. Upon infection, MVMp-infected cells accumulate in the S phase, while cells expressing NS1 are arrested in G_1, S, and G_2, with accumulation in the G_2 phase. In MVMp-infected cells, massive accumulation of the NS1 protein occurs in the S phase, since viral DNA amplification and P4 promoter activation occur in this phase (6, 8, 20). NS1 is thus massively synthesized when infected cells enter the S phase; it then interferes with cell DNA replication, arresting the cell cycle in this phase. Dex-induced clones, on the other hand, synthesize the NS1 protein in all phases. Various levels of NS1 accumulation could induce various numbers of nicks in the cell DNA and accordingly trigger early or late arrest of the cell cycle. The link between the abundance of cell chromatin lesions and their consequences is indeed known to depend on the phase (13).

In conclusion, we have characterized the cytostatic activity of NS1 and propose that it results from the generation of nicks in the cell chromatin. Whether NS1-induced nicks are produced through the endonuclease activity of NS1 itself or via activation of a cellular endonuclease remains to be determined. Whatever the case may be, the cytostatic effect of NS1 likely participates in the accumulation of MVMp-infected cells in S phase, providing an ideal environment for the virus, whose amplification depends on the host replication machinery. By preventing cell DNA replication, the virus gains uncompeted access to the cell synthetic machinery.

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ADDENDUM IN PROOF

Our results are supported by recent data published by M. B. Oleksiewicz and S. Alexandersen (J. Virol. **71:**1386–1396, 1997), who showed that infection by the autonomous parvovirus Aleutian mink disease virus causes a cell cycle arrest which is at least partly mediated by NS proteins. The interference of proteins of many viruses with the cell cycle is beginning to be extensively studied and was recently reviewed (A. Op De Beeck and P. Caillet-Fauquet, Viruses and the cell cycle, *in* L. Meijer, S. Guidet, and M. Philippe, ed., *Progress in Cell Cycle Research,* vol. 3, in press. Plenum Press, New York, N.Y.).

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