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The Cytotoxicity of the Autonomous Parvovirus Minute Virus of Mice Nonstructural Proteins in FR3T3 Rat Cells Depends on Oncogene Expression

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The nonstructural (NS) proteins of the autonomous parvovirus minute virus of mice are involved in viral DNA replication and in the regulation of homologous and heterologous promoters. Moreover, NS products have proved to be cytotoxic, especially for transformed cells. We show here that intracellular accumulation of NS products is not sufficient to kill rat fibroblasts from the established cell line FR3T3, which is phenotypically normal in several respects. FRNS cell lines were obtained by stable transfection of FR3T3 cells by a vector carrying the NS genes under the control of the hormone-inducible long terminal repeat promoter of the mouse mammary tumor virus. In the presence of dexamethasone, the NS proteins were synthesized without associated cell death. Transformation of FRNS cells with the c-Ha-*ras* oncogene or polyomavirus oncogenes had little effect on their capacity for NS induction, as measured at both concentration and transactivating activity levels, yet the transformants were now dying within a few days in the presence of the inducer. The same results were obtained with cells stably transfected by a vector expressing the NS1 product alone, suggesting that in this system there is no cooperation between NS1 and NS2 for maximal cytopathic effect. Cell mortality after NS protein induction was quantitatively related to the yield of oncogene expression, while NS-1 was not limiting in this respect. Our results show that the NS1 protein is not lethal unless cellular factors that may depend on oncogene expression trigger its cytotoxicity.

Parvoviruses can infect numerous animal species, including humans. Interest in these agents arises mostly from the striking specificity of virus-host interactions. Parvoviruses contain a single-stranded DNA that replicates in the nuclei of infected cells during the S phase. The low genetic complexity of parvoviruses implies that they tightly depend on exogenous factors to achieve their lytic cycle. In autonomous parvoviruses like the minute virus of mice (prototype strain MVMp), the helper factors are provided by the host cell and are expressed as a function of cell proliferation and differentiation (8). These requirements, which reflect interactions between highly regulated cellular functions and viral determinants, are likely to account for the spectrum of cytopathogenicity of parvoviruses both in vivo and in cell cultures. The genome of MVMp is organized in two overlapping transcription units with promoters located at map units 4 and 38 (7, 22). The P4 promoter directs the synthesis of two transcripts that differ in their splicing patterns and are translated into the nonstructural (NS) phosphoproteins NS1 and NS2. The NS1 protein is a multifunctional polypeptide with a size of 83 kDa that is essential for viral replication and transcription. It regulates the activity of its own promoter and transactivates the P38 promoter that controls the synthesis of the capsid proteins VP1 and VP2 (9, 25, 26). The NS1 product also regulates some exogenous promoters (14, 27). The role of the NS2 protein during the virus life cycle is still elusive; it is required for efficient virus production (19) and viral mRNA translation (20) in murine cells and seems to act in synergy with NS1 for maximal parvovirus cytotoxicity in human neoplastic cells (4, 16).

Often isolated from tumor tissues or stocks of oncogenic viruses (34), parvoviruses were initially suspected of being oncogenic. However, subsequent investigations did not support this possibility. On the contrary, epidemiological and experimental studies have shown that parvoviruses are able to prevent the appearance or cause the regression of a large variety of tumors (28). Therefore, the frequent association of parvoviruses with tumor tissues seems to reflect an opportunistic relationship instead of a causal one (29). Consistently, a number of phenotypically normal cells resisting parvovirus infection proved to become susceptible to parvovirus replication and the killing effect after in vitro transformation (6, 17, 18). This suggests that neoplastic transformation may act by triggering cellular factors involved in parvovirus development and/or cytopathic effect. It is noteworthy that the accomplishment of a complete viral lytic cycle is not necessary to kill transformed cells (13) and, conversely, that the production of infectious viral particles can be dissociated from cell killing (31). It has been shown that oncogenic transformation of established rat cells correlates with a parallel increase in their sensitivity to the cytocidal effect of MVMp and their capacity to synthesize the parvovirus NS proteins (6, 15, 30, 37). This and other indirect evidence (4, 21, 26) suggested that NS proteins may have a cytotoxic activity. The direct implication of NS proteins in cell killing has been demonstrated in human neoplastic cells which were stably transfected with the MVMp NS transcription unit cloned under the control of the hormone-inducible long terminal repeat (LTR) promoter of the mouse mammary tumor virus (LTR-NS). After induction of

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NS protein synthesis by dexamethasone (Dex), cell transfectants showed a typical cytopathic effect and died after a few days (5). These experiments indicate that the accumulation of NS products can lead to cell killing, although sensitivity to a given concentration of NS proteins appears to vary from one cellular clone to another.

In order to identify cellular modulators of NS cytopathogenicity, we investigated whether cells resisting MVMp infection were killed when they were forced to synthesize NS products. To this end, we used the established rat cell line FR3T3, which is phenotypically normal in many respects, to obtain derivatives that have integrated the LTR-NS construct and can be induced to synthesize the NS proteins by incubation with Dex. Some of these FRNS lines were subsequently transformed with various oncogenes and were compared with the parental lines for their NS production capacities and their survival in the presence of the inducer. Parental cells were found to survive intracellular levels of NS proteins that were lethal for the transformed derivatives, implying a direct or indirect effect(s) of oncogenes on the cytotoxic potency of NS polypeptides.

MATERIALS AND METHODS

Plasmids. Plasmid pSV2neo carries the aminoglycoside phosphotransferase gene conferring resistance to geneticin (G418) (35). Plasmid Py3 harbors the hygromycin B phosphotransferase marker, which allows the selection of hygromycin B-resistant clones after transfection in eucaryotic cells (2).

Plasmid pSV2neoEJ contains the c-Ha-*ras* oncogene from the human bladder carcinoma cell line EJ (32) at the *Bam*HI site of pSV2neo and was a gift of C. Marshall (London, United Kingdom). Plasmid pPyMT1 carries the coding sequence for polyomavirus middle T antigen (24), while pPyMLT97 is a hybrid plasmid encoding separately polyomavirus middle T antigen and the amino-terminal part of the large T antigen (1).

Plasmid pULB3232 contains the MVMp genome in which the P4 early promoter has been replaced by the hormone-inducible LTR promoter of the mouse mammary tumor virus (5). pULB3238 is a derivative of pULB3232 in which the capsid protein genes have been partly deleted (LTR-NSΔVP) (5). Plasmid pULB3278 differs from pULB3238 by a total deletion of the capsid genes and by three mutations in the splicing donor site for NS2, which prevents the synthesis of this product (LTR-NS1ΔVP) (16). Plasmid pULB3562 (pP38CAT) contains the chloramphenicol acetyltransferase (CAT) reporter gene under control of the P38 promoter of MVMp (5).

Cells. The NB 3205 cell line is a derivative of the simian virus 40 (SV40)-transformed newborn human kidney line NBE, in which pULB3232 (LTR-NS) is stably integrated (5). These cells were grown in minimal essential medium supplemented with 5% fetal calf serum (Boehringer).

The FR4 cell line, a subclone of the FR3T3 established line of Fisher rat cells (33), was described elsewhere (15). FREJ4 (37) and FRMTT4 (24) are FR3T3 cell derivatives transformed by the c-Ha-*ras* and polyomavirus middle T oncogenes, respectively. The FRNS and FRNS1 clones were selected by cotransfecting FR4 cells with the pSV2neo marker together with pULB3238 (LTR-NSΔVP) or pULB3278 (LTR-NS1ΔVP), respectively. The c-Ha-*ras*-transformed derivatives were isolated after FRNS or FRNS1 cell cotransfection with the Py3 marker and pSV2neoEJ. The polyomavirus transformants were obtained by infecting FRNS or FRNS1 cells with wild-type polyomavirus (50 PFU per cell) and subcloning a number of morphologically transformed clones that developed on the monolayer. Polyomavirus middle T or middle-plus-large T oncogene-transformed derivatives were selected after cotransfection of FRNS1 cells with the Py3 marker and pPyMT1 or pPyMLT97, respectively. All FR cells were grown in Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum and 1% sodium pyruvate.

DNA transfer. Transfections were performed according to a previously described modification of the calcium phosphate precipitation method (4).

For transient expression assays, cells were transfected with plasmids carrying the CAT reporter gene, maintained for 24 h, further incubated with or without 10^{-5} M Dex (stock solution in ethanol) for 24 h, and harvested. CAT activities were determined as described by Gorman (11). Cell extracts were adjusted to the same amount of proteins in each assay.

For stable transformation, transfections were carried out with the selection marker pSV2neo or Py3 and a threefold molar excess of the plasmid to be integrated. Respective selection with G418 (Gibco [0.8 mg/ml]) or hygromycin B (Sigma [0.4 mg/ml]) was started 2 days after transfection, and surviving colonies were recovered 8 to 12 days later.

Cell growth and survival. For the measurement of cell growth, cultures (5.10^4 cells per 60-mm-diameter dish) were incubated in the absence or presence of Dex (10^{-5} M). Every day, two plates of each culture were trypsinized and counted with a hemacytometer.

The survival of cells was determined by measuring their relative cloning efficiencies on plastic in the presence versus the absence of the inducer. Cells were seeded at a low density onto 60-mm-diameter dishes and incubated overnight at 37°C before adding (or not adding) Dex at a final concentration of 10^{-5} M. Colonies were stained and counted after 8 to 12 days.

Detection of the parvovirus NS proteins. (i) NS1 transactivating activity. NS1 transactivating activity was quantified by CAT assay, after cell transfection with the pP38CAT plasmid (see "DNA transfer").

(ii) NS1 accumulation. NS-1 accumulation was visualized by immunoblotting. Cultures (10^6 cells) were treated (or not treated) with 10^{-5} M Dex and incubated for 24 h. After trypsinization, 4.10^5 cells were lysed in 0.2% sodium dodecyl sulfate (SDS)–100 mM Tris-HCl (pH 6.8)–25% glycerol–0.2% bromophenol blue–20 mM β-mercaptoethanol. Proteins were fractionated by SDS–10% polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to a nitrocellulose membrane in 25 mM Tris-HCl–150 mM glycine–20% methanol (pH 7.9). Blots were incubated with a polyclonal rabbit antiserum raised against a bacterial fusion protein that contains an amino acid sequence specific for the carboxy-terminal region of MVMp NS1 (15). Immunocomplexes were revealed by means of anti-rabbit antibodies linked to alkaline phosphatase (Protoblot kit from Promega).

Detection of the p21^{ras} oncoprotein. (i) Immunoprecipitation and SDS-PAGE. Cultures (10^6 cells) were labelled by incubation in 2 ml of methionine-free Dulbecco's modified minimal essential medium supplemented with 200 μCi of [^{35}S]methionine (800 Ci/mmol [Amersham]) for a period of 18 h. Cells were lysed in 10 mM Tris-HCl (pH 7.4)–1 mM EDTA–150 mM NaCl–1% Triton X-100–0.5% sodium desoxycholate–1% aprotinin, and centrifuged at 100,000 × *g* for 1 h. Lysates were immunoprecipitated with the monoclonal pan-*ras* antibody FIII-85 (Oncogene Science, Inc.), fractionated by SDS-PAGE (15% polyacrylamide), and processed for fluorography.

(ii) Western blotting (immunoblotting). After incubation with 10^{-5} M Dex for 24 h, cultures (10^7 cells) were lysed at 4°C in 1 ml of 10 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 7.4) containing 5 mM EDTA, 100 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, 15 mg of aprotinin per ml, 0.1% SDS, and

0.5% sodium desoxycholate. The homogenates were treated for 20 min at room temperature with 0.2 mg of DNase I (Sigma) per ml in the presence of 30 mM $MgCl_2$, and centrifuged for 5 min at 10,000 × *g*. Extracts from 5.10^5 cells were submitted to SDS-PAGE (12% polyacrylamide) and electrophoretically transferred to a nitrocellulose paper for 2 h at 200 mA in Tris-glycine buffer containing 20% methanol. Blots were saturated with 3% bovine serum albumin (BSA) for 1 h at 37°C, rinsed three times for 15 min in TBST (10 mM Tris-HCl [pH 8], 150 mM NaCl, 0.05% Tween 20), and incubated overnight at room temperature with the mouse monoclonal pan-*ras* Val12 antibody DWP (Oncogene Science, Inc.) at 3 μg/ml in TBST containing 1% BSA. Detection was performed with anti-mouse antibodies linked to alkaline phosphatase (Protoblot kit from Promega).

Detection of the polyomavirus middle T oncoprotein (PyMT). After cell lysis, PyMT was immunoprecipitated according to the method of Salomé et al. (31) by using polyclonal antibodies obtained by intraperitoneal injection of polyomavirus-transformed PyB4A cells into BN rats (a gift of T. Benjamin, Boston, Mass.). Recovered PyMT was labelled by an in vitro kinase reaction in the presence of [γ-^{32}P]ATP (5 μCi per sample, 10 Ci/mmol [Amersham]), as described by Gelinas et al. (10). Samples corresponding to about 10^6 cells were adjusted to the same amount of total proteins, fractionated by SDS-PAGE (10% polyacrylamide), and visualized by autoradiography.

RESULTS

Establishment and in vitro transformation of LTR-NS stable transfectants from FR3T3 cells. In order to study the influence of cell transformation with oncogenes on the cytotoxic action of NS proteins, we used a system in which the parvovirus products were conditionally expressed. FR4, a subclone of the established FR3T3 cell line, was chosen for its low cloning efficiency in soft agar (less than 10^{-6}) and high resistance to MVMp infection (around 80 to 100%). FR4 cells are phenotypically normal, apart from the fact that they are immortal and have a high capacity for forming colonies on plastic. These cells were cotransfected with pULB3238 (LTR-NSΔVP) and the pSV2neo plasmid, allowing transfection-competent cells to be selected for their resistance to G418 (35). Twenty-seven G418 (800 μg/ml)-resistant cellular clones were isolated and amplified. The presence and functioning of integrated NS genes were checked through the ability of the NS1 protein to transactivate the MVMp P38 promoter linked to a reporter gene. To measure the basal and induced levels of NS proteins, cells were transfected with pP38CAT, induced (or not induced) with 10^{-5} M Dex 24 h later, and quantified for CAT expression 48 h after transfection. The percentage of acetylation of ^{14}C-labelled chloramphenicol reflected the activity of NS proteins in stably transfected cells. Thirteen G418-resistant clones displayed significant NS1 activity upon Dex treatment and were designated FRNS. The respective levels of induction ranged from 5-fold to 100-fold, which may be explained by interclonal differences in the number and/or genomic localization of the LTR-NS inserts. Four independent FRNS clones (110, 111, A21, and A33) were chosen for further analysis on the basis of their relatively high level of NS induction by Dex (Fig. 1B).

The FRNS clones were put through a procedure of oncogenic transformation by SV40, polyomavirus, and the c-Ha-*ras* oncogene. To achieve SV40 and polyomavirus transformation, nonconfluent cultures were infected with virus at a multiplicity of infection of 50 PFU per cell. A number of clones of morphologically transformed and piling-up cells were isolated from polyomavirus-treated monolayers and subcloned, giving rise to the so-called FRNSPy lines. Interestingly, we failed to isolate stable SV40 transformants from FRNS cells, although such derivatives could be readily obtained from the FR4 parent (see Discussion). Transformation by the c-Ha-*ras* oncogene was achieved by cotransfection of FRNS cells with pSV2neoEJ and the Py3 plasmid carrying the hygromicin resistance marker gene (2). A series of hygromycin B (400 μg/ml)-resistant clones with a transformed phenotype were picked up and amplified, generating the FRNSEJ cell lines. Because the results were similar for transformants selected from the four different FRNS clones, only those obtained with FRNSA33 derivatives are shown.

Effect of NS gene induction on the survival of parental and transformed cells. The fates of parental and transformed rat cells that have integrated the LTR-NSΔVP construct were determined after NS gene induction. The survival of cells was expressed as their relative cloning efficiency on plastic in the presence versus absence of Dex. As shown in Fig. 1A, the four FRNS clones tested were highly (92 to 98%) resistant to the inducing treatment, under conditions in which the survival of NB3205 human cells (used as a positive control) was reduced to 2%. In contrast, all tested polyomavirus- and c-Ha-*ras*-transformed derivatives of FRNSA33 cells proved to be sensitive to the inducer, although to a variable extent (5 to 51% survival). This sensitization was dependent on the presence of integrated NS genes because polyomavirus- and c-Ha-*ras*-transformed derivatives of FR3T3 cells (designated FRPy1 and FREJ4, respectively), which contained no NS insert, were resistant to incubation with Dex. The drop in survival of FRNS transformants in the presence of Dex is therefore likely to be a reflection of the cytotoxicity of induced NS proteins rather than a side effect of the drug on the capacity of transformed cells for scoring in the assay.

It was then investigated whether the sensitivity of transformed cellular clones to the toxic effect of the inducing treatment was correlated with a variation of their level of NS protein production. As shown in Fig. 1B, the polyomavirus and c-Ha-*ras* transformants derived from the FRNSA33 clone were all competent for the induction of NS proteins, as assessed from their ability to transactivate the P38 promoter in the presence of Dex. Oncogenic transformation was not associated with a major change in the capacity of the cell lines for NS1-mediated transactivation. Nevertheless, the selected c-Ha-*ras*- and polyomavirus-transformed derivatives exhibited significantly higher and lower NS1 activities than the parental FRNSA33 line, respectively. This difference was confirmed by quantification of NS1 protein accumulation by Western blotting, by using a monospecific antiserum directed against this product. As illustrated in Fig. 2A and in agreement with CAT assays mentioned above, the NS1 protein was recovered in somewhat higher amounts from c-Ha-*ras*-transformed versus parental FRNSA33 cells, whereas it tended to be less abundant in the polyomavirus-transformed derivatives. This variation was likely to reflect a differential action of polyomavirus and Ras oncoproteins at the level of the LTR promoter programming the transferred NS genes. Indeed, the capacity of a polyomavirus middle T oncogene-transformed derivative of FR3T3 cells (FRMTT4) for transient expression of a pLTR-CAT reporter plasmid, in the presence of Dex, was lower than that of the parental line, whereas this capacity was greater for the c-Ha-*ras*-transformed FREJ4 cells (data not shown). It is noteworthy that the promoter of the c-Ha-*ras* oncogene is itself stimulated by glucocorticoids (see reference 36 and Fig. 6A).

These results indicate that the differential toxicity of NS

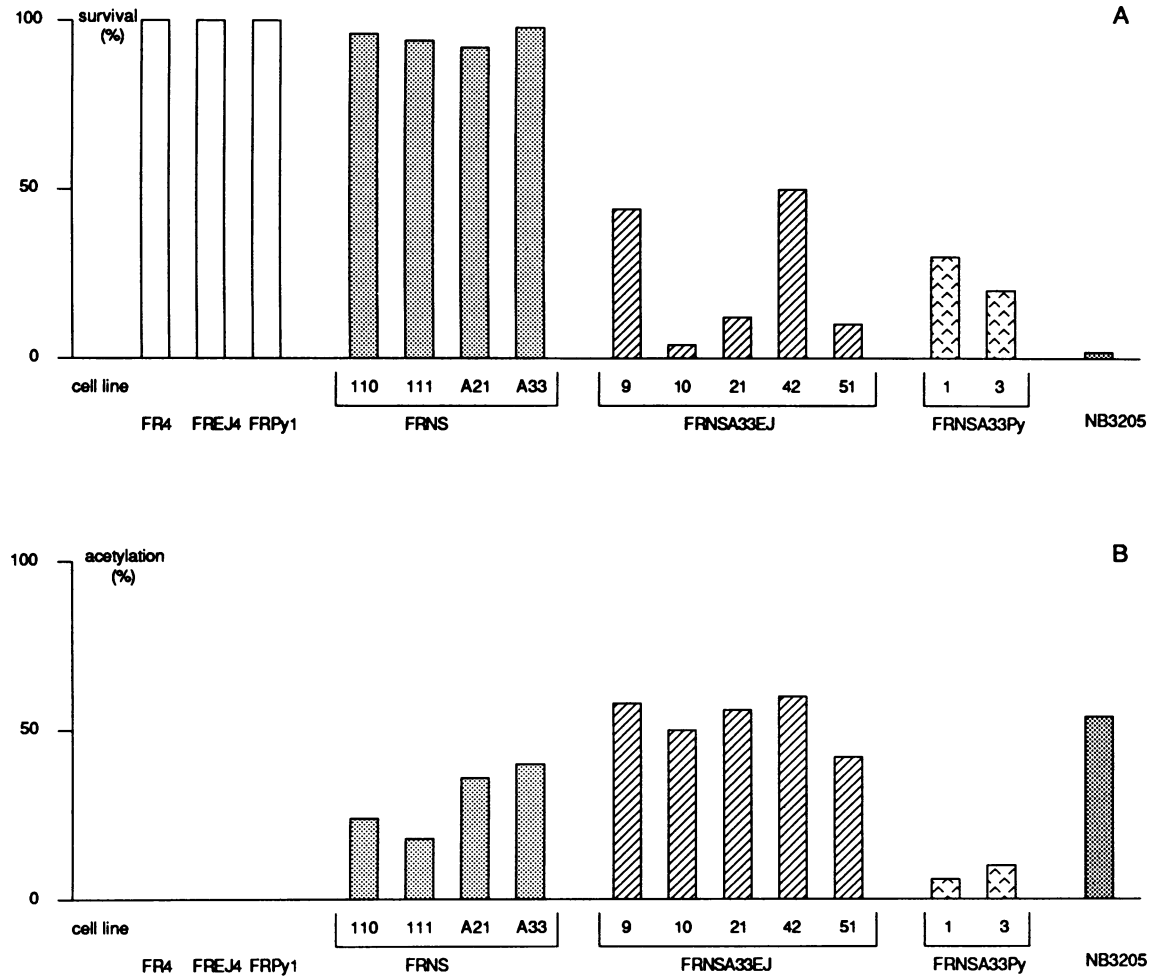

FIG. 1. Survival and NS1 transactivating activity of FRNSA33 cells and transformed derivatives. (A) Cell survival was calculated as the relative cloning efficiency of 10^{-5} M Dex- versus mock-treated cells. The cloning efficiencies in the absence of Dex ranged between 70% and 100% for all cell lines. (B) NS1 transactivating activity was measured by CAT assay after 24 h in the presence of 10^{-5} M Dex. The percentage of total ^{14}C-labelled chloramphenicol in acetylated forms was determined after cutting out and counting the chromatography spots. The uninduced levels of transactivation ranged between 0.3% and 1.5%. Data shown are average values from three experiments (standard deviation, <20%).

proteins observed after induction of parental cells and transformed derivatives was not due to different levels of NS protein synthesis. As is apparent from Fig. 1A, the sensitization to induced killing affected all transformed cells, including the FRNSA33Py lines, which accumulated fewer NS products than their FRNSA33 parent (Fig. 2A). In addition, the transformants showed interclonal variations in sensitivity, which did not correlate with NS concentration or activity (Fig. 1 and 2A).

Because the MVMp molecular clone used to establish the FRNS system (pULB3238) contains a partial deletion in the capsid genes and is deficient in replication (3), the results mentioned above confirm that NS proteins can have a cytotoxic effect in the absence of viral DNA amplification and functional capsid protein synthesis. It was reported that in neoplastic human cells, NS1 is sufficient to cause a major cytopathic effect, while NS2 seems to be required for maximal cytotoxicity (4, 16). In murine cells, NS2 is needed during the lytic cycle for efficient virus production (19). The question therefore arose as to whether induction of the NS1 product alone would also lead to the preferential killing of transformed versus parental rat fibroblasts. To this end, the present work was extended to FR4 derivatives that were stably transfected with plasmid pULB3278. This plasmid carries mutations in the splice donor site for NS2, which prevents the synthesis of this product, as shown previously (16). Among the stable transfectants obtained in this way, the FRNS1-25 line was selected for its high level of NS1 transactivating capacity after Dex treatment (Fig. 3B). Transformed derivatives of FRNS1-25 were obtained, as described above, by infection with polyomavirus (FRNS1-25Py) or cotransfection with the Py3 selection plasmid and molecular clones of the c-Ha-*ras* (FRNS1-25EJ), polyomavirus middle T (FRNS1-25MT), or polyomavirus middle-plus-large T (FRNS1-25MLT) oncogene. As shown in Fig. 3A, the parental FRNS1-25 cells were highly (98%) resistant to NS1 induction, while all transformed lines were sensitive to a certain degree (0.5 to 36% survival). As in the case of the FRNS system, the sensitization of FRNS1-derived transformants could not be ascribed to an increase in their concentration or activity of NS1 proteins (Fig. 2B and 3B). It is noteworthy that for the limited number of cellular clones studied, the killing induced by NS1 in FRNS1-Py or -EJ derivatives (Fig. 3A) was at least as severe as that achieved by

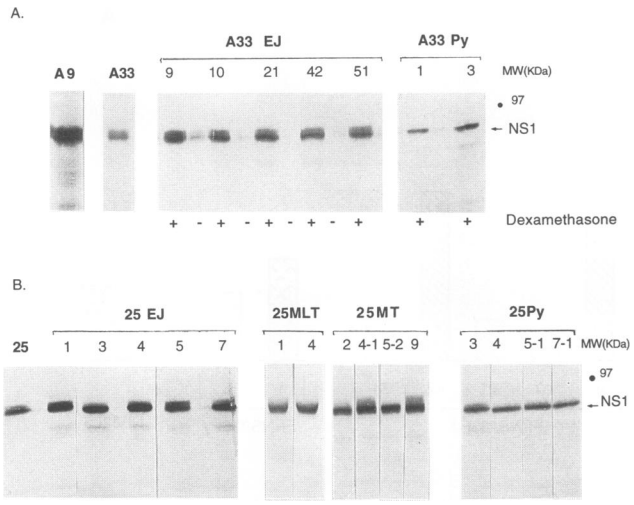

FIG. 2. Immunodetection of the NS-1 protein. Samples (2.10^5 cells) from cultures treated for 24 h with 10^{-5} M Dex were processed for protein extraction, fractionation, blotting, and NS1 detection by immunostaining with alkaline phosphatase. (A) FRNSA33 line and transformed derivatives compared with MVMp-infected mouse A9 cells used as a positive control. (B) FRNS1-25 line and transformed derivatives. MW, molecular mass protein markers. Arrows, positions of the NS1 product.

NS1 plus NS2 in FRNS transformants (Fig. 1A) and that for similar levels of NS1 (Fig. 2) and $p21^{ras}$ (see below). These data indicate that the NS1 product is a self-sufficient and major—if not unique—effector of parvovirus cytotoxic activity in transformed rat fibroblasts. As illustrated in Fig. 4, the transformed FRNS1 clones showed, after a few days in the presence of the inducer, a typical cytopathic effect characterized by the appearance of large abnormal cells with irregular shapes and disrupted nuclei. These disturbances were not detected in induced parental FRNS clones, which were indistinguishable from untreated ones except for a slightly reduced size. The selective toxicity of NS1 proteins was confirmed by the analysis of the growth properties of cells in which NS1 synthesis was or was not induced. Figure 5A shows that the presence of Dex in the medium did not significantly affect the growth of NS-free cells, whether parental FR3T3 or c-Ha-*ras* transformed (FREJ4). Therefore, the overexpression of the transferred c-Ha-*ras* oncogene in Dex-treated EJ transformants mentioned above was not reflected in a detectable change at the level of their proliferation in culture. This allowed us to use this system to determine whether the growth of c-Ha-*ras*-transformed cells was impeded when Dex additionally induced the production of NS1 proteins. In the absence of Dex, cells carrying an LTR-NS1 insert (clone FRNS1-25 and its c-Ha-*ras*-transformed derivative FRNS1-25EJ5) were found to display slightly reduced growth rates and saturation densi-

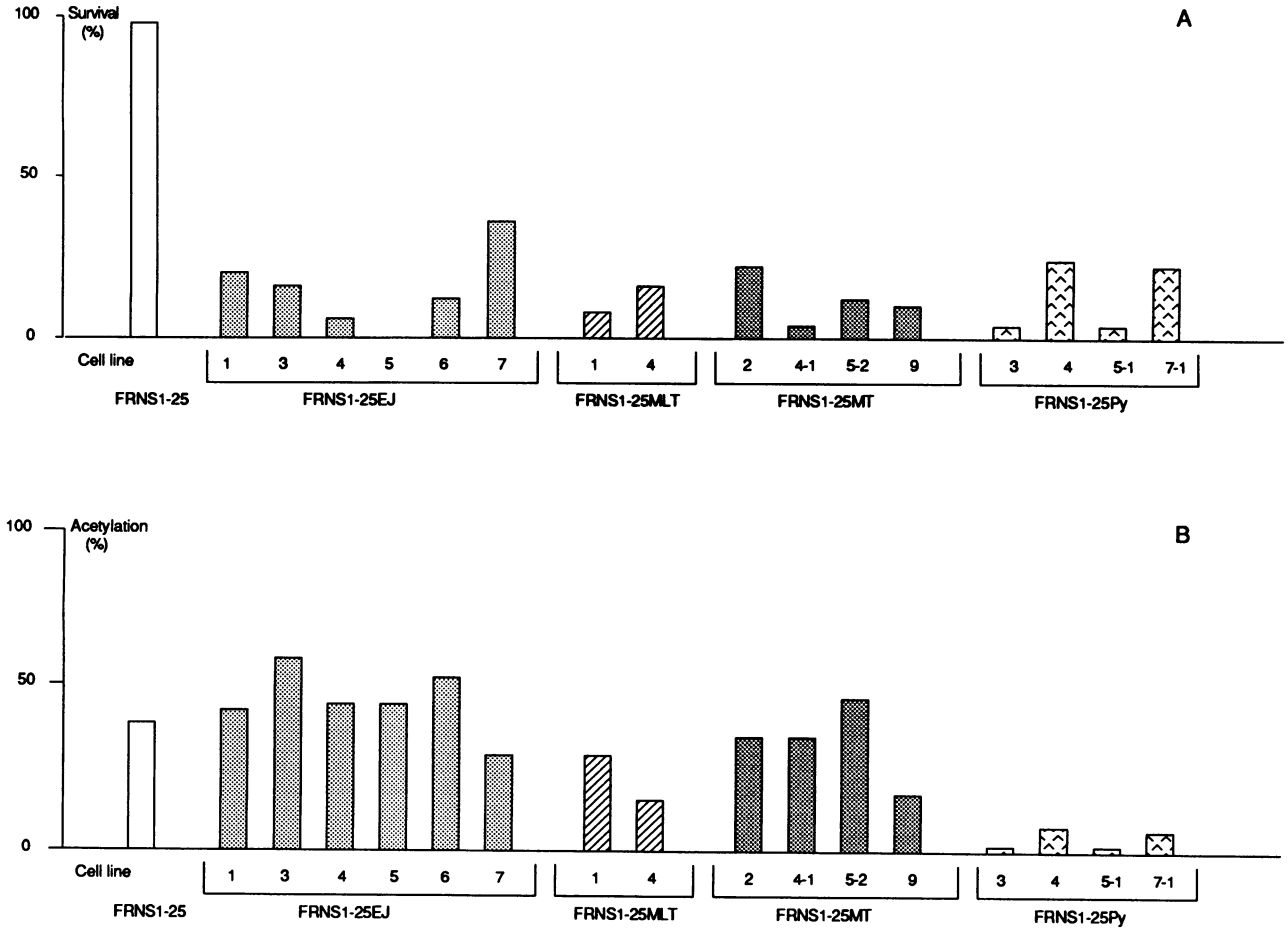

FIG. 3. Survival and NS1 transactivating activity of FRNS1-25 cells and transformed derivatives. Cells were analyzed as described in the legend to Fig. 1. Data shown are average values from two experiments (standard deviation, <20%).

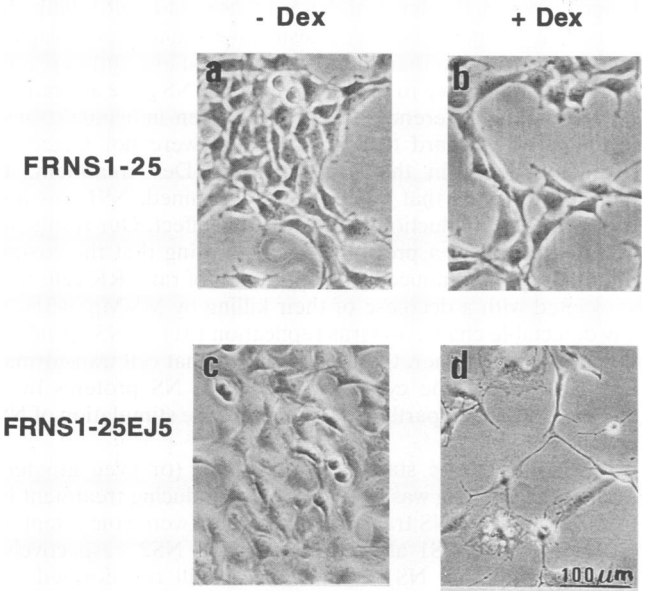

FIG. 4. Morphological appearance of parental and c-Ha-*ras*-transformed FR cells sustaining conditional NS1 gene expression. Phase-contrast micrographs of FRNS1-25 cells (a and b) and c-Ha-*ras*-transformed FRNS1-25EJ5 derivatives (c and d) are shown. Cell cultures were treated (b and d) or not treated (a and c) with 10^{-5} M Dex for 3 days.

ties compared with the FR3T3 and FREJ4 lines (Fig. 5). When the NS1 gene was induced by Dex, the parental FRNS1-25 cells grew more slowly but reached the same plateau as the noninduced cultures. This is in agreement with the cloning efficiency experiments, which showed that, in the presence of Dex, the number of clones was hardly affected but their size was reduced. On the contrary, induction of NS1 in transformed FRNS1-25EJ5 cells had dramatic consequences for their survival because the number of cells per plate decreased until day 6. The recovery occurring afterwards could be explained by the outgrowth of residual Dex-resistant variants. Altogether, our results indicate that the toxicity of NS1 protein in FR3T3 cells depends on oncogenic transformation.

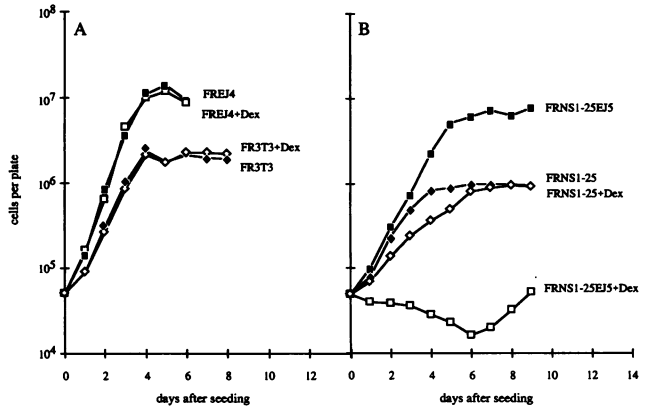

FIG. 5. Effect of NS1 induction on the growth of FRNS1-25 cells and the c-Ha-*ras*-transformed derivative FRNS1-25EJ5. (A) Control NS protein-free cells. Normal FR3T3 (diamonds) and c-Ha-*ras*-transformed FREJ4 (squares) cultures were incubated in the absence (solid symbols) or presence (open symbols) of 10^{-5} M Dex. FREJ4 cells became detached from the substrate after 6 days in culture. (B) Inducible NS transfectants. FRNS1-25 cells (diamonds) and FRNS1-25EJ5 cells (squares) were grown without (solid symbols) or with (open symbols) 10^{-5} M Dex.

Relationship between NS1 cytotoxicity and oncogene expression. As stated above, no quantitative correlation was found between the killing effect of NS induction in the various transformed clones and the level of induced NS proteins. This feature may be explained by interclonal variations in transformed cell factors that modulate the cytotoxic activity of NS products. It was more particularly investigated whether cell responsiveness to NS1 toxicity may be related to the extent of transformation and/or oncogene expression.

The various transformants could not be distinguished by their cloning efficiencies in soft agar, which all ranged between 45% and 55% (data not shown). Yet, the comparison of clones transformed by a given oncogene revealed a fair correlation between their respective sensitivities to NS induction and the levels of immunodetected oncoproteins. Among c-Ha-*ras*-transformed FRNSA33 derivatives, the three most sensitive ones (EJ10, EJ21, and EJ51 [Fig. 1A]) were also characterized by their level of $p21^{ras}$, which was higher than those of the other clones (Fig. 6). Similarly, the c-Ha-*ras*-transformed FRNS1-25 lines that were most susceptible to NS1-induced killing were the best $p21^{ras}$ producers. In particular, the FRNS1-25EJ5 derivative showed both the highest sensitivity to NS1 (0.5% survival) and the greatest $p21^{ras}$ expression, while accumulating NS1 at a level similar to that of FRNS1-25EJ7 cells, which were more resistant (36% survival) and less proficient in $p21^{ras}$ production (Fig. 2B, 3A, and 6C). Furthermore, the FRNS1-25MT2 clone could be distinguished from the other MT transformants by both its lower sensitivity to NS1 induction (Fig. 3A) and its lower capacity for p56MT expression (Fig. 6D). Altogether, these correlations raised the intriguing possibility that oncoproteins were directly or indirectly involved in the achievement of the cytotoxic action of NS1 in FR3T3 rat fibroblasts.

DISCUSSION

A number of established and nonestablished cell strains resisting MVMp infection are found to be sensitized to the parvovirus cytotoxic effect as a result of their transformation by various oncogenes (28, 29). The transformants show a concomitant increase in their ability to replicate and to express the viral genome. In particular, the sensitivity of c-Ha-*ras*-transformed derivatives of FR3T3 cells to MVMp infection proves to vary in parallel to their capacity for NS protein synthesis (37). Therefore, the resistance of corresponding parental cells to the killing effect of parvoviruses may be explained by their weak competence in NS protein synthesis. It was suggested that oncogenic transformation makes cells capable of producing high enough amounts of NS polypeptides for death to ensue (37).

The present report indicates that this view is oversimplified and that an additional effect of transformation needs to be considered other than the stimulation of NS production, namely, the sensitization of target cells to the toxic effect of a given level of NS proteins. Using the FR3T3 line of rat fibroblasts, we isolated a series of stable transfectants that had integrated the NS genes under control of the hormone-inducible LTR promoter of the mouse mammary tumor virus. When these FRNS lines were induced to produce NS proteins in the presence of Dex, the viability of the cells was not significantly affected. Although intracellular NS levels varied

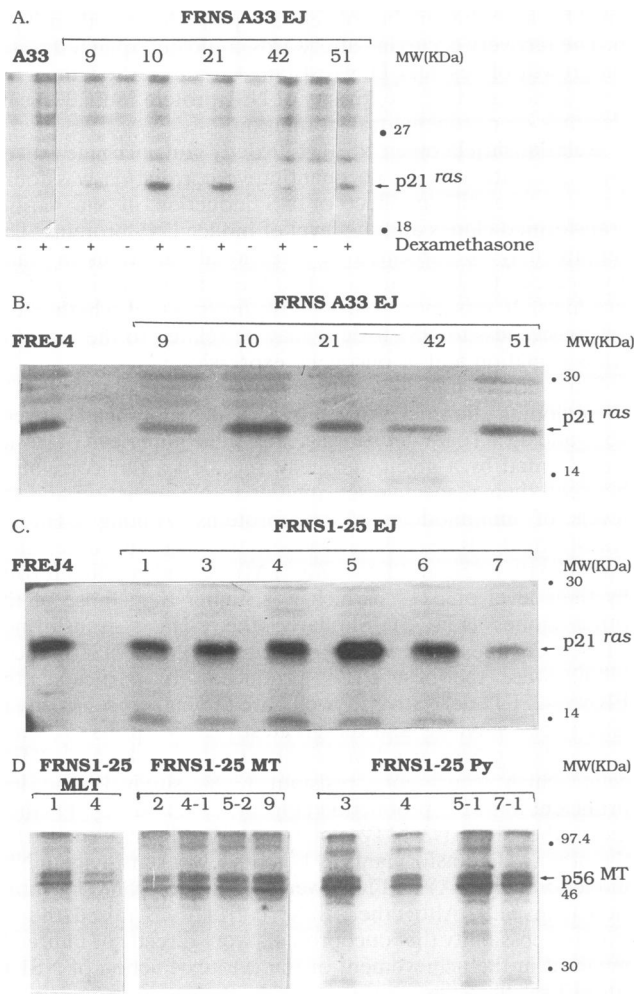

FIG. 6. Immunodetection of oncoproteins. (A) Fluorogram of $p21^{ras}$ immunoprecipitates from [^{35}S]methionine-labelled FRNSA33 cells and c-Ha-*ras* (EJ)-transformed derivatives. Samples from cultures treated (+) or not treated (−) with 10^{-5} M Dex for 24 h were adjusted to the same amount of ^{35}S-labelled proteins. (B) Western blotting detection of $p21^{ras}$ in c-Ha-*ras*-transformed FRNSA33 EJ derivatives. Samples (5.10^5 cells) from cultures treated with 10^{-5} M Dex for 24 h were processed for $p21^{ras}$ separation and immunostaining with alkaline phosphatase. The c-Ha-*ras*-transformed FREJ4 cell line was used as a positive control. (C) Western blotting detection of $p21^{ras}$ in c-Ha-*ras*-transformed FRNS1-25EJ clones. Samples were processed as described for panel B. (D) Autoradiogram of in vitro-phosphorylated PyMT immunoprecipitates. Middle-plus-large T (MLT) or middle T (MT) oncogene and polyomavirus (Py)-transformed derivatives of FRNS1-25 cells were induced with 10^{-5} M Dex for 24 h. Samples were adjusted to equivalent amounts of total proteins. MW, molecular mass protein markers. Arrows, positions of $p21^{ras}$ (A, B, and C) and $p56^{MT}$ (D).

between different clones, some transfectants produced NS1 to an extent that was found to reduce the viability of human neoplastic NBE cells to 2%. In contrast, NS induction proved to be cytotoxic for all FRNS derivatives that had been subsequently transformed with the tumor virus polyomavirus or isolated oncogenes of viral (Py) or cellular (c-Ha-*ras*) origin. These derivatives accumulated different levels of NS proteins, depending on the transforming agent and undetermined factors responsible for interclonal variations. No correlation was found between the level of NS synthesis and cell killing. In particular, the polyomavirus transformants had a lower capacity for NS induction than the FRNS parental line and were still markedly susceptible to the killing effect of NS gene activation. Moreover, the differences detected between individual transformants with regard to NS production were not linked to parallel changes in their sensitivity to Dex treatment. It therefore appears that in the clones examined, NS1 was not limiting for the induction of a cytopathic effect. Our results fit well with those of a previous report showing that the partial reversion of v-*src*-induced transformation of rat NRK cells was associated with a decrease of their killing by MVMp, without any detectable change in virus replication (31) or NS1 production (16a). Altogether, these data suggest that cell transformation may trigger the cytopathogenicity of NS proteins by a mechanism at least partly independent of the stimulation of NS production.

It should also be stated that a similar (or even greater) extent of cell killing was achieved by the inducing treatment in FRNS1 versus FRNS transformants, which were competent in the synthesis of NS1 alone and NS1 plus NS2, respectively. This indicates that NS1 is sufficient to kill transformed rat fibroblasts and confirms other reports showing that this protein is the major effector of parvovirus toxicity in transformed human cells (4, 16). While NS2 was found to reinforce the cytotoxic activity of NS1 in the latter system, no cooperative effect of NS2 was detected in the FR transformants.

The mode of action of NS1 is still poorly understood. NS1 is a multifunctional protein that plays an essential role in viral DNA replication and the regulation of parvovirus and heterologous promoters (9, 14, 26). There is no evidence so far for a direct interaction of NS1 with promoter sequences (12), suggesting that this protein may act by associating with cellular proteins that participate in promoter recognition and/or regulation, possibly through its terminal acidic amino acid sequences (23). A recent site-directed mutagenesis study failed to dissociate the transcriptional functions of NS1 from its cytopathic potential (14). Therefore, it can be speculated that NS1 may jeopardize cell survival by activating or inhibiting suicide or vital cellular genes, respectively.

In conclusion, we have shown that the NS1 protein is not cytotoxic when expressed in FR3T3 fibroblasts. This could not be due to a mutation in the transferred NS1 gene, since NS1 proteins retained their transactivating ability and, more importantly, became lethal after cell transformation. It remains to be determined whether transformation affects the activity of NS1 through the modification of the viral product and/or the availability of cellular cofactors or targets. Whatever that may be, the sensitization of transformed cells to NS1-induced killing may depend on their capacity for oncoprotein production, as suggested by the parallel variation of both parameters among clones transformed by a given oncogene (PyMT or c-Ha-*ras*). In contrast, NS1 did not appear to be limiting in the induced transformants tested, in so far as their killing is concerned. Our failure to isolate SV40 transformants from FRNS cultures in the absence of Dex mentioned above could be interpreted along that line, i.e., the small amount of glucocorticoids normally present in the serum would induce a basal level of NS proteins that is lethal for cells transformed by this potent oncogenic agent. These considerations raise the possibility that putting sensitive cultures of polyomavirus or c-Ha-*ras* oncogene-transformed FRNS cells through a procedure of selection for resistance to Dex treatment may lead to the isolation of variants impaired in NS1 and/or oncoprotein production or activity. This work is in progress and should help

to unravel the interconnection of regulatory networks involving oncogene products and parvovirus NS proteins.

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