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# Programmed killing of human cells by means of an inducible clone of parvoviral genes encoding non-structural proteins

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**Although its dependence on the target cell type is well established, the cytopathogenicity of parvoviruses has remained elusive to date as far as its mechanism is concerned. However, indirect evidence suggested that parvoviral non-structural (NS) proteins may be the cytotoxic effectors. In order to test this hypothesis, a molecular clone of parvovirus MVMp was modified, by replacing the P4 promoter of the NS transcription unit by the glucocorticoid-inducible promoter of the mouse mammary tumour virus. Clones of neoplastic human cells that had incorporated this construct and that were induced to produce NS proteins by dexamethasone, showed a cytopathic effect and eventually died. Our data strongly suggest that the intracellular accumulation of parvoviral NS products jeopardizes the survival of the cells, which cannot be detected unless a threshold protein concentration is reached. Interestingly, a cell variant could be isolated which resisted dexamethasone-induced killing, although it was fully inducible for the production of NS proteins. This variant was also unusually resistant to infection with MVMp virions, thus confirming the essential role played by the NS proteins in the parvoviral cytotoxicity and indicating that the cytocidal activity of the parvoviral NS products is modulated by cellular factors that may vary from one cell to another.**

*Key words:* induced cytotoxicity/mouse mammary tumour virus promoter/non-structural proteins/parvovirus minute virus of mice

## Introduction

Parvoviruses are small non-enveloped lytic viruses whose genome consists of a linear single-stranded DNA molecule and which contaminate a variety of animals, including humans. The low genetic complexity of these viruses may account for their tight dependence on exogenous factors for their replication. These factors can be provided by helper viruses (Berns and Bohenzky, 1987) and/or the host cell (Cotmore and Tattersall, 1987). Interestingly, the expression of some of these cellular helper functions appears to be conditional on cell proliferation and differentiation (Cotmore and Tattersall, 1987; Yakobson *et al.*, 1987). These requirements are likely to contribute to the striking specificity of parvoviral infections, as demonstrated in cell cultures (Guetta *et al.*, 1986; Antonietti *et al.*, 1988; Gardiner and Tattersall, 1988) and reflected back *in vivo* by the pathogenicity of these viruses (Siegl, 1984; Studdert, 1990). The specificity of parvovirus-host cell interactions is exemplified by the oncotropism of a number of these viruses and may underlie their capacity for preventing the appearance or causing the regression of a variety of tumours (Rommelaere and Tattersall, 1990). In addition, the expression of a transformed phenotype proved to correlate with an increase in the sensitivity of certain cells to the cytopathic effect of parvoviruses, suggesting that neoplastic transformation may be included among the conditions modulating cell susceptibility to these viruses (Salomé *et al.*, 1989).

Although its dependence on the target cell type is well established, the cytopathogenicity of parvoviruses remained elusive up to date as far as its viral effector(s) and mechanisms are concerned. However, recent results indicate that the so-called non-structural proteins of parvoviruses, designated NS or Rep and involved in the regulation of viral DNA replication and expression (Cotmore and Tattersall, 1987; Berns and Bohenzky, 1987), may disturb host cells. Thus, NS/Rep proteins appear to interfere with a series of non-parvoviral processes, including gene expression programmed by heterologous promoters (Labow *et al.*, 1987; Rhode and Richard, 1987), initiator-induced DNA amplification (Heilbronn *et al.*, 1989), *in vitro* neoplastic transformation (Hermonat, 1989) and stable cell transformation with exogenous DNA (Labow *et al.*, 1987; Rhode, 1987; Ozawa *et al.*, 1988; Brandenburger *et al.*, 1990). These data suggest that the NS gene products encoded at least by the three studied viruses H1, MVM and B19, may have a cytotoxic activity. This possibility would be consistent with the fact that oncogenic transformation of rat cells correlates with a parallel increase in their sensitivity to the killing effect of parvovirus MVMp and in their capacity for viral NS protein synthesis (Van Hille *et al.*, 1989). However, it cannot be ruled out that the parvoviral inhibition of DNA transformation reflects the interference of NS proteins with the integration of the transforming gene or its long-term stability in the cellular genome.

The aim of the present work was to remove these uncertainties concerning the nature of the parvoviral cytotoxic effectors. To this end, an infectious molecular clone of parvovirus MVMp (Merchlinsky *et al.*, 1983) was modified by replacing the original promoter of the NS transcription unit, P4 (Cotmore and Tattersall, 1987), by the glucocorticoid-inducible heterologous promoter, the long terminal repeat (LTR) of the mouse mammary tumour virus (MMTV) (Hynes *et al.*, 1983). These MMTV-MVMp chimeric constructs were used to obtain clones of transformed human cells in which the parvoviral NS genes were

permanently present in a silent form and could be turned on upon treatment with dexamethasone. The analysis of this conditional system demonstrates in a direct way that the intracellular accumulation of NS proteins can eventually result in cell death. These data raise the possibility of using parvoviral NS genes to induce the killing of cells whose destruction is desirable, such as neoplastic cells.

## Results

In an attempt to assess the cytotoxicity of NS proteins, the NS-coding region of an infectious molecular clone of MVMp, pMM984 (Merchlinsky *et al.*, 1983), was placed under the control of a constitutively weak but inducible promoter derived from the mouse mammary tumour virus (MMTV-LTR). This promoter responds to glucocorticoid stimulation in transfected NB-E cells. Indeed, expression of a *cat* reporter gene programmed by the MMTV-LTR promoter (Cato *et al.*, 1986) was induced approximately 100-fold by dexamethasone, a synthetic glucocorticoid (data not shown).

### *Characterization of pMMTV-LTR-NS plasmids by transient expression assays*

Two plasmids were constructed in which the P4 promoter of MVMp is replaced by the MMTV-LTR promoter: pULB3238 has a 594 bp deletion in the capsid genes whereas pULB3232 contains intact VP genes (see Materials and methods). In order to determine the basal and induced levels of expression of the NS genes under control of the MMTV-LTR promoter in both plasmids, we have taken advantage of the ability of NS-1 proteins to transactivate the late parvoviral P38 promoter (Rhode and Richard, 1987; Doerig *et al.*, 1988). Thus, NB-E cells were cotransfected with pMMTV-LTR-NS (pULB3232, pULB3238) and pP38cat (pULB3562) plasmids. The level of transactivation of CAT expression, determined by the percentage of acetylation of $^{14}$C-labelled chloramphenicol, reflects the activity of NS proteins in transfected cells.

Figure 1 shows a comparison of the levels of NS proteins expressed either from the legitimate parvoviral promoter P4 (plasmid pMM984), or from the mock- and dexamethasone-induced MMTV-LTR promoter (plasmid pULB3238). Dexamethasone had no detectable effect on the activity of the P38 promoter but triggered NS expression from the MMTV-LTR in both pULB3238 (Figure 1) and pULB3232 (data not shown) plasmids. The extent of stimulation of CAT activity (denoting NS induction by dexamethasone) was considerable (about 60-fold) and similar for both chimeric clones. Yet MMTV-LTR-driven production of NS proteins in the presence of dexamethasone did not reach the level achieved by the P4 promoter.

### *Establishment of transformed cell lines expressing NS proteins from the MMTV promoter*

In order to demonstrate the cytotoxicity of the NS proteins directly, we set up a cellular system allowing their conditional expression. NB-E cells were cotransfected with either MMTV-NS construct and with the pSV2neo plasmid carrying a selectable marker gene. 13 geneticin (G418)-resistant cellular clones were isolated and amplified, out of which six had been inoculated with pULB3232 and seven with pULB3238. To measure the basal and induced levels of NS proteins in these DNA transformants, CAT expression was quantified in the different cellular clones 48 h after cell supertransfection with the pP38cat plasmid. P38 transactivation could not be detected in 7 of the cellular clones analysed, whether they were exposed to the inducing agent or not. The other 6 strains displayed a significant NS activity upon dexamethasone treatment, although their respective levels of induction ranged from 2- to 95-fold. Representative cellular clones are shown in Figure 2 (columns A and B). This variability may be due to interclonal differences in the number and/or genomic localization of functional integrated copies of the MMTV-NS plasmid.

In parallel, NB-E cells were cotransfected with pMMTV-LTR-cat (Cato *et al.*, 1986) and pSV2neo. Among eleven G418-resistant clones, 7 proved to be inducible for CAT expression in the presence of dexamethasone (Figure 2, column C).

### *Survival of induced cell clones*

The cloning efficiency of the different DNA-transformed cell lines was measured in the absence or in the presence of the inducer. Dexamethasone was used at a final concentration ($10^{-5}$ M) that had no detectable effect on the clonogenicity of parental NB-E cells (data not shown). In a control experiment, 11 NB-E clones, transformed with the MMTV-LTR-cat plasmid, were tested for their sensitivity to dexamethasone. They formed colonies in the presence of the drug, irrespective of the extent to which they were prone to induction (Figure 2, panel C). Actually, under the cell culture conditions used, some transformants (obtained with MMTV-LTR-cat as well as MMTV-LTR-NS) formed larger and more numerous colonies (survival $>100\%$) when incubated with dexamethasone (data not shown in Table I). In contrast, two out of the 13 transformed cell strains obtained with the MMTV-NS recombinant plasmids, showed a severe reduction (50-fold or more) in their cloning ability upon treatment with the inducer (Figure 2, panels A and B). These same two cell strains, NB3205 and NB3894, displayed a particularly high level of NS protein induction (Figure 2, panels A and B). These observations are therefore consistent with a role of parvoviral products in the inhibition of cell clonogenicity.

NS proteins are known to regulate the synthesis of parvoviral DNA replicative intermediates and capsid proteins positively (Cotmore and Tattersall, 1987; Rhode and Richard, 1987). However, the particular MVMp molecular clone used for the present constructions does not replicate, due to a small deletion in its non-coding 5′ terminal part (Boissy and Astell, 1985; F.Dupont, personal communication). Moreover, the pULB3238 plasmid in cell strain NB3894 does not encode functional capsid proteins (Figure 2, panel B). Therefore, the inhibition of cell clonogenicity apparently does not require the production of parvoviral DNA or capsids and is very likely to be due directly to the NS polypeptides, although a contribution of the N-terminal region of VP proteins cannot be ruled out.

NB3205 and 3894 cells were plated at low density, induced to synthesize NS proteins and examined microscopically after the addition of dexamethasone. This analysis revealed a clear cytocidal effect of NS proteins. Interestingly, cell lysis occurred only after 5–7 days of continuous dexamethasone induction, when microcolonies had already formed (3 or 4 cell doublings). If cultures were subconfluent

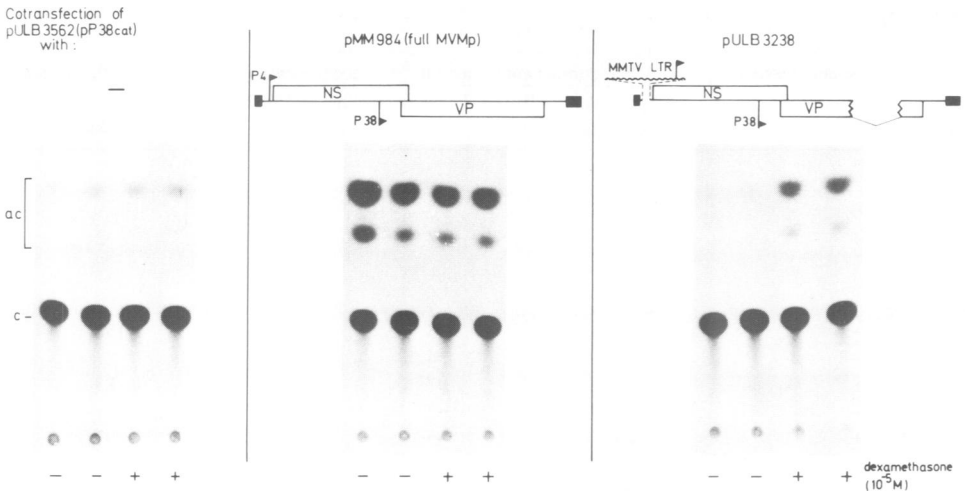


**Fig. 1.** Evaluation of constitutive and inducible MVMp molecular clones for the production of NS proteins in transient expression assays. NB-cells were transfected with the pULB3562 plasmid (pP38cat), conjointly with MVMp molecular clones whose NS region is controlled by the genuine parvoviral promoter P4 (pMM984) or the glucocorticoid-inducible MMTV-LTR (pULB3238) (see simplified genetic maps and Materials and methods). Dexamethasone ($10^{-5}$ M) was added 24 h after transfection and cells were further incubated for 24 h in the presence of the inducer. CAT assays were then performed to determine NS production, owing to the capacity of NS proteins for transactivating the parvoviral P38 promoter in pULB3562. Positions of non-reacted chloramphicol (c) and two acetylated derivatives (ac) are shown. Symbols: ■, terminal palindromic sequences; ⚑, transcriptional start site; NS, NS protein-coding region; VS, capsid protein-coding region.

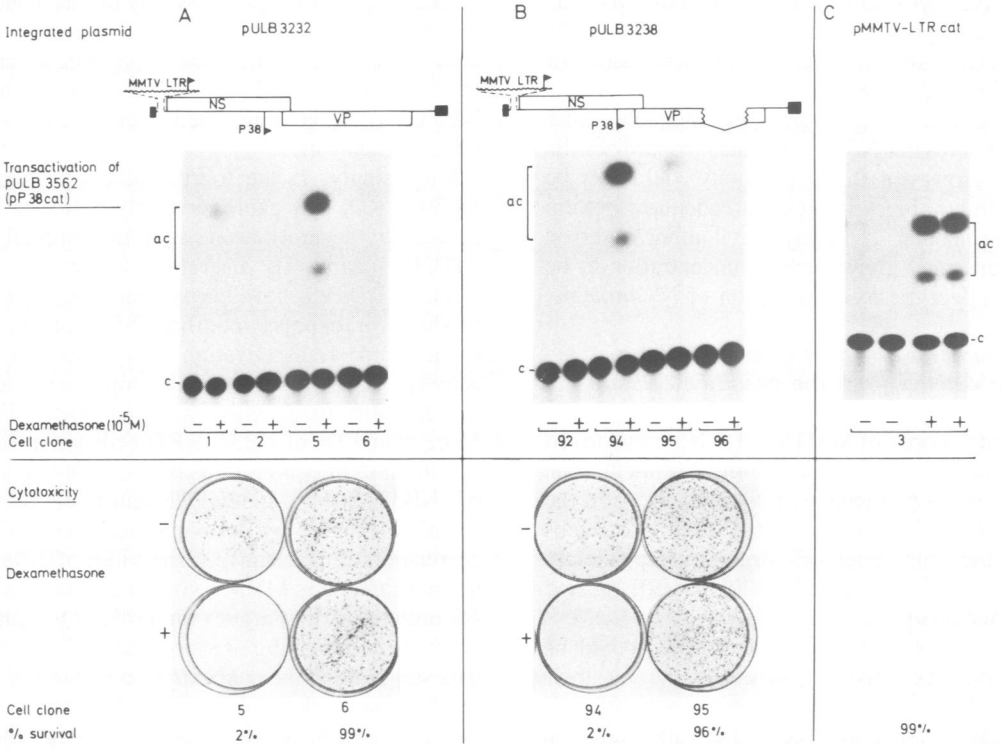


**Fig. 2.** Characterization of stable cell transformants expressing NS proteins from the inducible MMTV promoter.(**A**,**B**) Analysis of cells transfected by the NS-inducible MVMp molecular clones pULB3232 (A) and pULB3238 (B) containing intact and deleted capsid genes, respectively (see simplified maps and Materials and methods). For the evaluation of NS induction (upper panels), cells were transfected with the reporter plasmid pULB3562 and tested for CAT activity after addition of dexamethasone, as described in Figure 1. For the evaluation of NS cytotoxicity (lower panels), the formation of colonies from single cells was measured in the presence or absence of dexamethasone. Cell survival was calculated as the relative cloning efficiency of drug- versus mock-treated cells (average values from three experiments; standard deviations less than 20%). Total numbers of surviving colonies were 53, 3133, 22, 2941 and 435 for clones 5, 6 (A), 94, 95 (B) and 3 (C), respectively. Clones 3 and 4 (A) and 91, 93 and 97 (B) are not shown and were all non- or poorly inducible for NS production and fully resistant to the inducing treatment. Clones 5 (A) and 94 (B) are labelled as NB 3205 and NB 3894 in the main text. (**C**) A strain of cells permanently transformed with a pMMTV-LTR-cat construct (Cato *et al.*, 1986) was tested for the induction of CAT activity (upper panel) and cell survival (lower panel) upon treatment with dexamethasone. Similar results were obtained with 6 other independently isolated cell transformants (not shown). Symbols used are the same as in Figure 1.

**Table I.** Analysis of dexamethasone-resistant subclones of the sensitive cell strain NB3205

| Cell clones | Dexamethasone treatment[a] | NS transactivation activity[b] (% acetylation) Resident pULB3232, Superinfecting pULB3232 | | Cell survival[c] (%) Dexamethasone induction | MVMp infection |
|---|---|---|---|---|---|
| Parental strain | | | | | |
| NB3205 | − | 2.5 | 7 | | 0.1 |
| | + | 83 | 97 | 2 | |
| Resistant subclones | | | | | |
| NB3205/RA | − | 1.5 | 4 | | 0.1 |
| | + | 1 | 95 | 136 | |
| NB3205/RB | − | 1 | 4.5 | | ND |
| | + | 2 | 97 | 83 | |
| NB3205/RC | − | 1 | 7 | | ND |
| | + | 23 | 95 | 93 | |
| NB3205/RD | − | 3 | 4.5 | | 41 |
| | + | 82 | 99 | 57 | |

[a]Induction with dexamethasone ($10^{-5}$ M) was as described in Figures 1 and 2.
[b]NS protein-mediated transactivation of the parvoviral P38 promoter was measured by CAT assays using the reporter plasmid pULB3562 (pP38cat), and is expressed as percentages of chloramphenicol acetylation. Cells were supertransfected, or not, with the same plasmid as the one used to achieve their stable transformation (pULB3232).
[c]Cell survival was calculated from the cloning efficiency of dexamethasone-induced or MVMp-infected cells and is expressed as % of mock-treated cells. The multiplicity of infection with MVMp was 5 plaque-forming-units per cell.
ND, not determined.

at the time of induction, growth continued to confluence and then a progressive cytopathic effect was observed, as characterized by the appearance of abnormal swollen cells with irregular shape and abundant cytoplasm (data not shown; Friedman *et al.*, 1989).

Altogether, these data strongly suggest that the intracellular accumulation of parvoviral NS polypeptides jeopardizes the survival of cells. However, this cytotoxicity could only be detected with cellular clones in which an adequate protein concentration was attained. The delay in cell killing observed in our experiments may allow such a concentration to be reached and/or reflect the mode of action of NS proteins.

### *Isolation and characterization of dexamethasone-resistant clones derived from the sensitive cell line NB3205*

Although the great majority of MMTV-LTR-NS-transformed NB3205 cells died in the presence of dexamethasone (Table I), a very small percentage of colonies survived the inducing treatment and were recovered after a 2 weeks incubation with the drug. Four cell strains, independently selected in this way and designated NB3205/RA/RB/RC and /RD, were further analysed.

Three of these selected resistant cell variants could be distinguished from the NB3205 parental line by their complete (NB3205/RA and /RB) or partial (NB3205/RC) inability to express the NS-associated transctivating function when exposed to dexamethasone (Table I). The impairment of NS induction by dexamethasone in these strains apparently does not occur at the level of hormone receptors. Indeed, when they were supertransfected with an exogenous MMTV-LTR-NS construct (pULB3232) together with the P38cat indicator plasmid, resistant cell clones proved to be fully proficient in NS induction (Table I). Therefore, the failure of these three inducer-resistant cell strains to express NS activity, in the presence of dexamethasone, may be ascribed to the loss or (epi)genetic alteration of their MMTV-LTR-NS inserts. Recently, it has been reported by Friedman *et al.* (1989) that an integrated MMTV-LTR-driven *gCI* gene of herpes simplex virus progressively lost its inducibility during successive cell subcultures *in vitro*. Such a drift was not noticed in our system, since no significant changes in NS induction and cell killing were observed when NB3205 and NB3834 cells were analysed over 20 successive passages (data not shown).

Surprisingly, in the fourth inducer-resistant cell clone, NB3025/RD, NS expression proved to be as efficiently induced by dexamethasone as in the parental sensitive line NB3205 (Table I). Therefore it can be assumed that NB3205/RD cells have become intrinsically resistant to NS products or express modified NS proteins retaining the capacity for transactivation in the absence of cytocidal activity. This resistant cell strain was tested for its susceptibility to infection with wild-type MVMp virions. More than 40% of NB3205/RD cells survived a parvoviral inoculum (5 infectious units per cell) that only spared 0.1% of NB3205 (dexamethasone-sensitive) or NB3205/RA (poorly inducible by dexamethasone) cells (Table I). The correlation of the relative sensitivities of different cell lines to both infectious MVMp virions and induced endogenous NS proteins further argues for a role of the latter parvoviral products in the killing of susceptible cells. Moreover, these data suggest that the concentration which NS proteins must reach to lead to detectable cellular lethality, may differ from one cell line to another and be particularly high in the NB3205/RD variant.

## Discussion

### *Cytotoxicity of parvoviral NS proteins*

Previous studies showed that the sensitization of a number of transformed cells to parvoviral infection correlated with an increase in their ability to replicate viral DNA and/or express viral genes encoding, in particular, NS proteins, although progeny virions were not necessarily produced (Cornelis *et al.*, 1988a; van Hille *et al.*, 1989). These

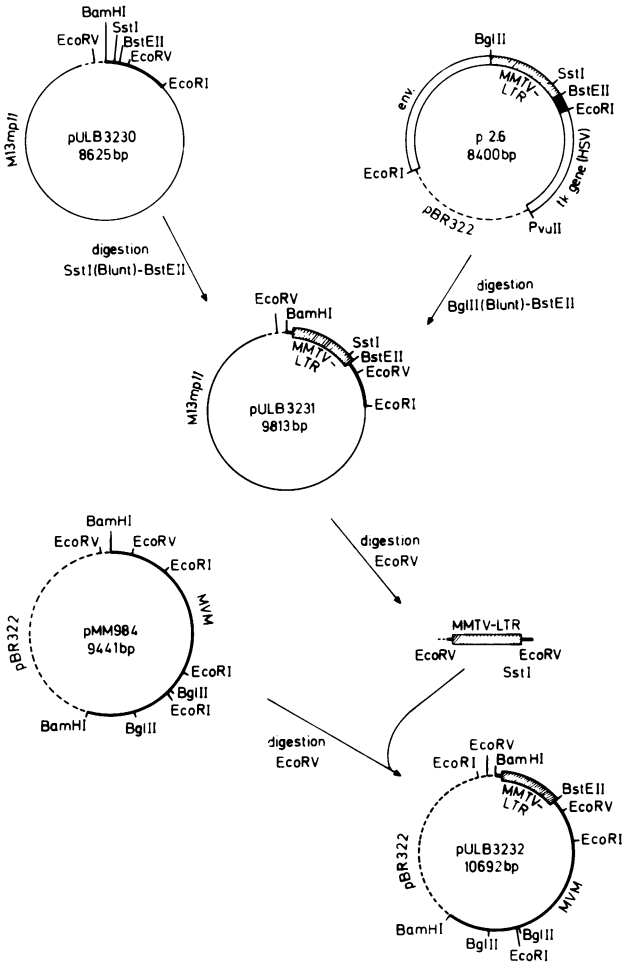


**Fig. 3.** Strategy for the construction of the MMTV-LTR-MVMp chimeric plasmid pULB3232. Successive steps are described in Materials and methods. Recombined DNA segments originate from plasmid pBR322, - - -; phage M13 mp11, —; parvovirus MVMp, — ; MMTV-LTR, ▨; mouse genomic DNA, ▩; and fragments of env and tk genes of MMTV and HSV viruses, □.

observations, together with the reported interference of molecular clones of parvoviruses with stable DNA transformation of cells (Labow *et al.*, 1987; Rhode, 1987; Ozawa *et al.*, 1988; Brandenburger *et al.*, 1990), raise the possibility that the non-structural proteins of these viruses may be endowed with a cytotoxic activity. The present paper provides direct evidence for this activity by showing that neoplastic human cells, which had incorporated a dexamethasone-inducible molecular clone of the MVMp non-structural region, died upon hormonal treatment. Dexamethasone was not responsible by itself for the observed cytopathic effect. Indeed, the drug rather stimulated the growth of cells that did not contain NS inserts or were poorly inducible for NS protein production, possibly by substituting for factors removed from the serum during its hormonal depletion. Cell killing did not require the amplification of MVMp DNA nor the expression of functional capsid proteins, but appeared to depend on the production of NS polypeptides. The MVMp genome encodes two partially overlapping non-structural proteins that are designated NS-1 and NS-2 (Cotmore and Tattersall, 1987). NS-1 appears to be essential for the disturbance of cells, although its cytopathic effect may be further reinforced by the NS-2 protein (Brandenburger *et al.*, 1990). The cytotoxicity of NS proteins does not seem to be restricted to NB-E cells since D.J.Pintel, L.K.Naeger, K.E.Clemens and G.Tullis reported at a recent meeting (EMBO workshop in the molecular biology of parvoviruses, Ma'ale Hachamisha Israel, November 1989) that the expression of these products similarly impaired the growth and reduced the plating efficiency of mouse A9 cells. It should be stated that these results do not exclude an additional cytotoxic action of capsid proteins or virions.

### *Varying cell susceptibility to NS proteins*

Interestingly, a variant (NB3205RD) could be isolated from a strain of dexamethasone-inducible and NS-sensitive cells. This variant proved to be fully inducible for NS production but to resist NS levels that were apparently intolerable to the parental cell strain. This observation, together with the resistance of the NB3205RD variant to MVMp infection, strongly suggest that the concentration of NS proteins, necessary for cytotoxicity to ensue, depends on the cell. Accordingly, cellular factors seem to modulate the cytopathogenicity of NS proteins and may occasionally vary as a result of the well known drift of established cell lines *in vitro* (Huschtscha and Holliday, 1983). It remains to be determined how the cellular modulators of NS cytoxocity act. Amongst numerous possibilities, they could modify NS proteins, interfere with their action, be the target for NS activity or serve themselves as killer (co)factors. In any case, the susceptibility of cells to the killing effect of parvoviruses appears to be determined not only by their ability to produce NS proteins but also by their intrinsic responsiveness to a given level of these polypeptides. It is presently unclear whether the responsiveness of target cells to NS products can be related to other phenotypic traits. A preliminary indication of such a relationship was provided by a recent report showing that the reversion of a temperature-sensitive transformed phenotype allowed the establishment of a persistant parvovirus infection in the absence of overt cytotoxicity (Salomé *et al.*, 1989). In this respect, it will be interesting to determine whether the NS-resistant cell variant identified in the present work can be distinguished from the parental line by other phenotypic differences and is able to produce progeny MVMp particles and release them by a non-cytocidal mechanism.

### *Cell killing mechanisms*

The mechanism by which NS proteins disturb target cells is presently unknown. It is well established that non-structural proteins participate in parvoviral DNA replication (Tullis *et al.*, 1988) and exert both positive and negative controls over homologous and heterologous transcription promoters (Rhode and Richard, 1987). Thus, NS proteins may conceivably interfere with the on-going replication and/or expression of cellular DNA, resulting in cytostatic and cytocidal effects. Yet, there is no proof so far that the nuclear translocation of NS proteins, known to occur in infected cells (Cotmore and Tattersall, 1987), is required for cytotoxicity to develop. Indeed, large T protein of SV40 is mainly nucleotropic but may exert part of its transforming effect in the cytoplasm (Smith, 1984). Cell killing is not limited to parvoviruses, or to lytic viruses, but is also achieved by certain immune-system cells and a number of bacteria, fungi and protozoan parasites. Some of these agents attack the

plasma membrane of target cells by secreting a lethal pore-forming protein (Young and Cohn, 1986). Assailed cells may also undergo an 'internal disintegration' triggered by a variety of processes, including the interruption of host protein synthesis and the perturbation of signalling pathways (Stephen and Pietrowski, 1986). Although their sites of action are presently unknown and may still involve the plasma membrane, NS proteins are more likely to operate according to the second cytotoxic mode. Indeed, the cytopathic effect due to NS proteins was not completed until several days after their induction and involved a progressive intracellular degeneration prior to the disruption of the plasma membrane. Yet comparison of sequences has not so far revealed a genuine homology between NS molecules and either pore-forming proteins or toxins (data not shown). Time-limiting steps of NS cytotoxicity may consist of the dilution or decay of an essential cellular factor(s) whose replenishment is prevented by the parvoviral products, the achievement of a critical level of NS-induced cellular damage or the intracellular accumulation of NS proteins themselves. It should be stated that the 'incubation period' prior to cell lysis was longer for cultures induced by dexamethasone (7–8 days) than for those infected with MVMp virions (1–2 days). The longer delay after NS induction may be ascribed to the lack of MVMp DNA amplification in DNA-transformed cells and/or to the somewhat lower activity of the activated MMTV-LTR promoter compared with that of the P4 promoter.

In conclusion, recent observations concerning both killer cells and lytic viruses indicate that there may be several different mechanisms responsible for the death of target cells, ranging from plasma membrane leakage to internal damage and extending over various periods of time. The recent identification and cloning of responsible genes from herpes (Friedman *et al.*, 1989) and parvoviruses (present work) will hopefully contribute to the characterization of some of these pathways and provide weapons to target toxic phenotypes in cells to be destroyed.

## Materials and methods

### *Cell line, virus and plasmids*

The SV40-transformed newborn human kidney cell line NB-E (Shein and Enders, 1962) was grown in MEM medium supplemented with 5% fetal calf serum (Boehringer). The serum was depleted of glucocorticoid hormones by dextran–charcoal treatment (Stanley *et al.*, 1977), in order to maintain the basal level of expression of the glucocorticoid-inducible promoter (MMTV-LTR) as low as possible. Parvovirus MVMp was propagated, purified and titrated by plaque-assay, essentially as described by Tattersall *et al.* (1976). Plasmid pSV2neo, carrying the aminoglycoside phosphotransferase gene (Southern and Berg, 1982) was used as a selective marker for stable transformation. pMMTV-cat (Cato *et al.*, 1986) was used as a control for the inducibility of expression from the LTR promoter in either transient or stable transformation assays. pMM984 is a recombinant plasmid containing the entire genome of MVMp (Merchlinsky *et al.*, 1983). The MMTV-LTR promoter was excised from the plasmid p2.6 containing the entire 3′ LTR of MMTV (Hynes *et al.*, 1983).

### *Measurement of cell cloning efficiency*

Infected or induced cells were tested for their survival and growth as described by Cornelis *et al.*, 1988a. To measure the cloning efficiency of MMTV-LTR-transformed cell lines upon induction, $5 \times 10^2$ to $10^3$ cells were plated onto 60 mm dishes and incubated at 37°C, overnight, prior to the addition of dexamethasone (Sigma, stock solution in ethanol) at a $10^{-5}$ M final concentration. Colonies were counted after 10 to 15 days incubation in the presence of dexamethasone.

### *DNA transformation assays*

Ca-phosphate transfections were performed as described (Brandenburger *et al.*, 1990). For transient expression assays, cells were recovered 48 h after transfection with plasmids carrying the reporter *cat* gene and chloramphenicol acetyltransferase (CAT) activity was determined as described by Gorman (1985). Cell extracts were incubated with $^{14}$C-labelled chloramphenicol during 2 h at 37°C. Protein concentrations were determined using the Biorad protein kit. Volumes of extracts were adjusted to give the same quantity of total proteins in each assay. For stable transformation assays, transfections were carried out with 1 μg of pSV2neo and a three-fold molar excess of MMTV-NS, or MMTV-CAT plasmids. Geneticin (G418, Gibco) was added at a final concentration of 0.7 mg/ml and surviving cell colonies were recovered 14 days after transfection.

### *Construction of new plasmids*

*pULB3545 (pP38).* The *Hae*III (1853)–*Pst*I (2129) fragment of MVMp DNA (Astell *et al.*, 1986), containing the P38 promoter and its transactivation region (tar) (Rhode and Richard, 1987), was inserted between the *Bam*HI and *Pst*I sites of the polylinker of pUC18 (Yammisch-Perron *et al.*, 1985). The *Bam*HI sticky end was filled in with the Klenow fragment of DNA polymerase I.

*pULB3562 (pP38cat).* The *Eco*RI–*Xho*I fragment, comprising the P38 promoter of MVMp, was recovered from pULB3545 and inserted between the *Sal*I and *Xho*I sites of pBGU7 (Cornelis *et al.*, 1988b). For this construction, the *Eco*RI end of the insert and the *Sal*I end of the vector were rendered blunt. The final plasmid contains the *cat* reported gene under the control of the P38 promoter of MVMp.

*pULB3232.* (i) The 1.4 Kb *Eco*RI fragment of pMM984, comprising the P4 promoter of MVMp, was cloned into the *Eco*RI restriction site of the M13mp11 vector (Messing and Vieira, 1982) to give plasmid pULB3221. The orientation of the insert was determined by digestion with *Pst*I or *Bam*HI endonucleases. (ii) The pULB3227 derivative was obtained by eliminating the 79 bp *Hin*dIII fragment containing the *Sst*I site of the polylinker from pULB3221. (iii) Site-directed mutagenesis was then performed on the single-stranded DNA of pULB3227. Two new restriction sites were created on either side of the P4 promoter by oligonucleotide-directed mutagenesis, using two synthetic 21mers. The first oligonucleotide introduces two mutations (T→G) in positions 134 and 136 (MVMp coordinates), creating a new *Sst*I restriction site. The second oligonucleotide introduces a single mutation (A→C) in position 246 (MVMp coordinate), creating a *Bst*EII restriction site. The resulting plasmid (pULB3230) is shown in Figure 3. (iv) The MMTV-LTR was obtained on a *Bgl*II–*Bst*EII fragment (1.3 Kb) from plasmid P2.6 (Hynes *et al.*, 1983). It was inserted, as a substitute for the P4 promoter of MVMp, into the *Sst*I–*Bst*EII sites of pULB3230, generating plasmid pULB3231 (see Figure 3). (iv) To replace the P4 promoter in pMM984 by the MMTV-LTR, the 1900 bp *Eco*RV fragment from pULB3231 was substituted to the 577 bp *Eco*RV fragment of pMM984, giving rise to the final plasmid pULB3232 (Figure 3). The orientation of the MMTV-LTR insert was ascertained by *Sst*I digestion.

*pULB3238.* Plasmid pULB3238 was obtained by removing the 694 bp *Bgl*II fragment from pULB3232, thus creating a deletion within the MVMp capsid genes.

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