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Construction of a Genetic Switch for Inducible *trans*-Activation of Gene Expression in Eucaryotic Cells

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The cotransfection of selectable marker genes and the gene for the nonstructural proteins NS1 and NS2 of the autonomous parvovirus H-1 failed to produce cell lines that constitutively expressed NS1. A plasmid, pP38NS1cat, was constructed that expressed the NS1-NS2 gene from the H-1 P38 coat protein promoter in place of the natural P4 promoter. The P38 promoter is constitutively weak and is *trans*-activated by NS1. Stable cell lines were isolated that contained pP38NS1cat that was constitutively silent, but inducible with exogenous NS1 by superinfection or by treatment with sodium butyrate. The cells that were induced for this self-stimulatory genetic circuit did not remain in the culture, suggesting that expression of NS1-NS2 is cytotoxic or that the expression is not sustained. The properties of these cell lines and an example of the construction of a cell line inducible for expression of the viral coat protein gene and the bacterial gene for chloramphenicol acetyltransferase (*cat*) are described.

The nondefective parvoviruses produce two nonstructural proteins, NS1 and NS2, which share the same gene and the same promoter (1, 2, 4, 5, 8, 11, 23, 24) (Fig. 1). The two capsid proteins, VP1 and VP2, also are derived from the same genomic region and appear to have a common promoter (1, 4, 5, 8, 11, 18, 23, 24). The promoter for NS1-NS2 is at map position 4, hence the name P4. The promoter of the capsid protein gene is located at map positions 38 to 40 in various parvoviruses and will be referred to in this study as P38. P38 is embedded within the structural gene for NS1. In kinetic studies, NS1 has been shown to appear earlier than the capsid proteins, although there was considerable overlapping of their syntheses (12, 17). I have reported evidence that NS1 positively regulates the expressiosn of P38, the promoter of the capsid protein genes, and that P38 has a very weak constitutive level of expression in the absence of NS1 (21). The dependo group of parvoviruses or adeno-associated viruses has been reported to have a similar regulation of their coat protein genes by their early proteins (10, 31). Because the parvoviruses have a single-stranded genome that has to convert to the double-stranded replicative-form DNA before viral protein synthesis can occur, it is not possible to divide the viral replicative cycle into early and late stages with metabolic inhibitors of DNA synthesis. With this caveat, parvoviruses seem to produce the nonstructural proteins NS1 and NS2 as early proteins and the capsid proteins as late proteins in a fashion analogous to that of other DNA viruses (9, 15).

To study the functions of parvovirus nonstructural proteins, it would be desirable to isolate and characterize viral mutants with altered NS1 or NS2 or both. To facilitate the propagation of such mutants, it would be convenient to have cell lines that produce these proteins and complement mutant viruses defective for their functions. Such cell lines were produced for adenoviruses (7). In this study, I describe both evidence that the parvovirus nonstructural proteins may be cytotoxic and a method for constructing cell lines with inducible expression of NS1. These cell lines exhibit the *trans*-activation function of NS1 on induction; therefore, they can have inducible expression of any cloned gene that is engineered to be expressed by the P38 promoter or a derivative of it. As an example of this application, I constructed a cell line that expresses the coat protein gene of a temperature-sensitive mutant of the parvovirus H-1 on induction.

MATERIALS AND METHODS

Cells. The simian virus 40-transformed human kidney cell line NB (26), HeLa Gey (ATCC CCL2.1), HeLa S3 (ATCC CCL2.2), and thymidine kinase-negative (tk^-) Rat 2 cells (30) were maintained in monolayer culture with Eagle minimal essential medium in the presence of 10% fetal calf serum and in the absence of antibiotics.

DNA constructions. A diagram of the plasmids is shown in Fig. 1. The plasmid pP38NS1cat was constructed by cloning the parvovirus H-1 P38 promoter region in the DNA fragment *Eco*RI-*Pst*I (nucleotides 1088 to 2132) into the plasmid pUC8. The pUC8 *Hin*dIII site was restricted and filled in with T4 DNA polymerase, and *Bam*HI linkers were added. After restriction with *Bam*HI, the plasmid, pH2B, was religated and recovered by transfection. This converted the *Hin*dIII site to a *Bam*HI site at the +127 position (relative to the cap site which is assumed to be nucleotide 2008) of the P38 promoter. The region −108 to +127 was recovered from this construction by restriction with *Nco*I-*Bam*HI and inserted into pH9catΔP4, which was derived from pH5cat (21), by the deletion from the *Bam*HI linker at the 5′ end of the H-1 sequences to the *Mbo*I site at nucleotide 1862 (−146 relative to the P38 promoter cap site) (Fig. 1). This plasmid, pP38a, has the pBR327 *Cla*I site at the 5′ side of the P38 promoter, does not have the *Bam*HI site that was at the left end of the genomic clone (21), and retains the *Bam*HI site at the +127 position. A *Bam*HI site was positioned at the start of the NS1 gene by first isolating the beginning of the gene from nucleotides 0 to 1250. This was done by deleting the plasmid pH5cat, which contains the complete H-1 genomic sequences from approximately 0 to the *Hin*dIII site at 2655 fused to the *cat* gene (21), of all the H-1 sequences and *cat* sequences 3′ to the *Bgl*II site at nucleotide 1250 to the *Bam*HI site after the simian virus 40 processing signals, (pP42; not shown). This rendered the *Nco*I site at the ATG start codon of NS1 a unique site. The *Nco*I site was filled in with T4 polymerase, and *Bam*HI linkers were added (pP42B2; Fig. 1). The P4 promoter was excised from the

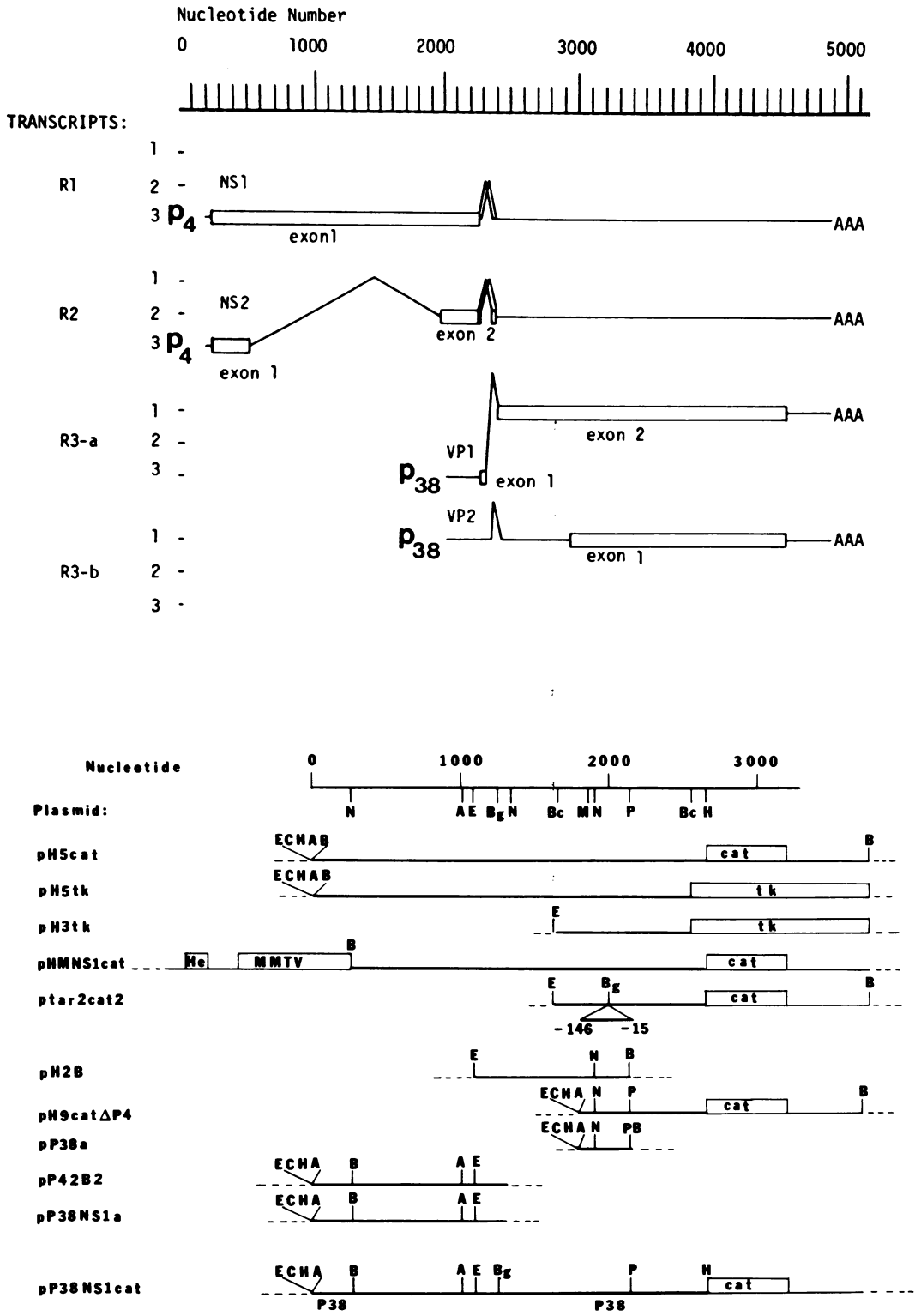

FIG. 1. Upper panel: The genomic organization of the autonomous parvoviruses. The transcripts of the P4 and P38 promoters are R1, R2, and R3-a, R3-b, respectively. The rows numbered 1, 2, and 3 represent the three reading frames. Only the translated portions of the transcripts have been designated exons in this figure. Lower panel: Diagram of plasmids. Line diagrams of plasmids used in this study are shown in alignment with their H-1 sequences in the upper panel. Bold lines, H-1 sequences; dashed lines, vector sequences. The restriction sites are: A, *Acc*I; B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; M, *Mbo*I; N, *Nco*I; and P, *Pst*I. Not all the sites to the indicated restriction enzymes are shown. pH5neo is similar to pH5cat except the 1.4-kilobase *neo* fragment is in the position of the *cat* gene. MMTV, mouse mammary tumor virus.

resulting plasmid with *Cla*I-*Bam*HI, and the *Cla*I-*Bam*HI fragment that contains the P38 promoter was ligated into its place (pP38NS1a). The P38 fusion to the 5′ portion of the NS1 gene was excised with *Cla*I-*Acc*I (*Acc*I is at nucleotide 1016) and ligated to a pH5cat plasmid, which had its *Bam*HI site 3′ to the *cat* gene previously destroyed. The resulting plasmid was pP38NS1cat (Fig. 1). The plasmid pH5tk was constructed by first forming a fusion between the P38 promoter and the *tk* gene. The *cat* gene was excised from pH3cat by restriction with *Bcl*I and *Bam*HI (the *Bcl*I site is at nucleotide 2561 in H-1) and replaced with the herpes simplex virus *tk* gene from the *Bgl*II site to the *Bam*HI site from ptk26 (pBR322 with the 2-kilobase *Pvu*II fragment of *tk* cloned with *Bam*HI linkers) to give pH3tk (Fig. 1). The 5′ end of the NS1 gene was obtained from pH5cat as an *Eco*RI-*Xba*I fragment and ligated into those sites of the pH3tk construction.

The plasmid pMNS1cat was constructed by removing the P38 promoter in front of NS1 in pP38NS1cat with *Cla*I-*Bam*HI and inserting the mouse mammary tumor virus promoter from the plasmid pM14-1 (16) as a *Cla*I-*Bam*HI fragment. The plasmid pHMNS1cat was constructed by inserting the Harvey sarcoma enhancer from the plasmid pM14-1 as a *Cla*I fragment into the *Cla*I site of pMNS1cat (Fig. 1).

The plasmid p2VP was constructed by ligating the *Hin*dIII-*Bam*HI fragment from the genomic clone of H-1 *ts*6 (21), which contains the coat protein region, to the *Hin*dIII-*Bam*HI-restricted plasmid ptar2cat2 (Fig. 1). Briefly, this plasmid has a tandem repeat of the P38 promoter with the 5′ copy including sequences from −146 to −15 and the second copy from −146 to +648 (*Hin*dIII site of H-1 at nucleotide 2655). ptar2cat2 has an increased constitutive expression of *cat* and a high *trans*-activation by NS1.

DNA transfections. DNA transfections were done as previously described (21). Calcium phosphate precipitates of plasmid DNA and carrier calf thymus DNA were added to 60-mm dishes of cells in a volume of 0.5 ml. The cultures were treated with 15% glycerol in HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer for 4 min at 4 h after transfection, and the cultures were subsequently treated with sodium-butyrate for 16 h as previously described (21). Cotransfections were done with the selectable marker genes aminoglycoside phosphotransferase (*neo*) in the plasmid pSV2neo (29) or guanine phosphoribosyltransferase (*gpt*) in the plasmid pSV2gpt (13).

NB cells were selected for *neo* gene expression by exposure to G418 at 1 mg/ml, and HeLa cells were treated with 1.5 mg/ml. The selections for *gpt* were as described previously (13). The selections for the herpes thymidine kinase (*tk*) gene were in medium containing 0.5 μg of methotrexate per ml, 10^{-5} M thymidine, nonessential amino acids as a source of glycine, and 13 μg of adenine per ml applied 48 h after transfection.

Transient expression assays. The activity of the reporter gene (*cat*) chloramphenicol acetyltransferase (CAT) was assayed as previously described (22). Samples with high activity were diluted until no more than 60% of the substrate was acetylated.

Immunofluorescent staining. Cells were plated on glass cover slips in 35-mm petri dishes and at the proper time were washed with Hanks balanced salt solution and fixed with cold acetone for capsid antigen staining and with 2.5% paraformaldehyde for 15 min at room temperature for NS1 staining. Dried and fixed cells were stored at −20°C until stained. Capsid antigens were detected with anti-H-1 antisera from hamsters that had survived a neonatal infection with H-1. The NS1-specific antibody provided by Sue Cotmore was produced by immunization of rabbits with an NS1 fragment fusion protein (5). The fixed slides were first incubated with 2% nonimmune hamster serum or 2% fetal calf serum before exposure to 2% dilutions of the specific antisera. After washing in phosphate-buffered saline, the primary antibody as detected by indirect immunofluorescence with 4% biotin-conjugated antirat antiserum for the hamster primary antibody or 4% biotin-conjugated antirabbit antiserum for the rabbit anti-NS1 antibody. Last, the slides were stained by incubation with a 4% dilution of the fluorescein-conjugated avidin. The biotin and avidin reagents were obtained from Vector Laboratory (Burlingame, Calif.). The micrographs were made with a Zeiss UV fluorescence microscope at ×25, ×63, or ×100.

TABLE 1. Inhibition of transformation of Rat 2 cells by cotransfection with plasmids containing the NS1-NS2 gene[a]

Plasmid	Amt (μg/dish)	Avg no. of tk^+ colonies/dish
Expt 1		
ptk26	1	40
ptk26 + pH5cat	1 + 5	1.5
ptk26 + pH3cat	1 + 5	43.5
Expt 2		
ptk26	1	188
ptk26 + pH5cat	1 + 4	32
ptk26 + pH5	1 + 4	26
ptk26 + pH6cat	1 + 4	52

[a] Rat 2 cells (2×10^5) were plated in 60-mm petri dishes and transfected the next day with ptk26 with and without various plasmids containing the NS1-NS2 gene. pH5cat (NS1-NS2 wild type) and pH6cat (truncated NS1–wild-type NS2) are as previously described (23). pH5 is a genomic clone of H-1 *ts*6 deleted in the coat protein gene from nucleotides 2657 to 4688. Two days after transfection the dishes were incubated with selective medium, and colonies were fixed, stained, and counted 12 days later. In some cases colonies were recovered before the fixation.

RESULTS

Toxicity of NS1-NS2 gene to stable transformation. In my initial experiments, I attempted to isolate stable cell lines that expressed NS1 and NS2 by cotransfection of a selectable genetic marker with plasmids that express these genes from their native P4 promoter. These plasmids were pH5cat and pH5neo. The results showed that a marked reduction in the yield of clones expressing the selectable marker occurred when the plasmids expressing NS1 and NS2 were included. The results of two such experiments are tabulated in Table 1. Rat 2 cells were transfected with ptk26 or cotransfected with ptk26 and NS1 plasmids. When pH5cat or pH5 was included, there was a reduction of greater than 80% in the number of tk^+ clones in many experiments. The plasmid pH6cat (21), which has a frameshift insertion of 4 base pairs at nucleotide 1250 within the intron of NS2 and makes a truncated NS1 and wild-type NS2, was partially inhibitory. In similar experiments, the plasmid pH5neo, which expresses aminoglycoside phosphotransferase, was very inefficient in generating G418-resistant clones compared with pSV2neo, even though in transient assays with the *cat* counterparts, pH5cat expressed higher levels of the reporter gene than did pSV2cat (21). pH5neo also reduced the yield of tk^+ clones when *tk* was the selectable marker gene (data not shown). Cotransfection of the *tk* plasmid with pH3cat, a fusion of only P38 to the *cat* gene, showed no inhibition of

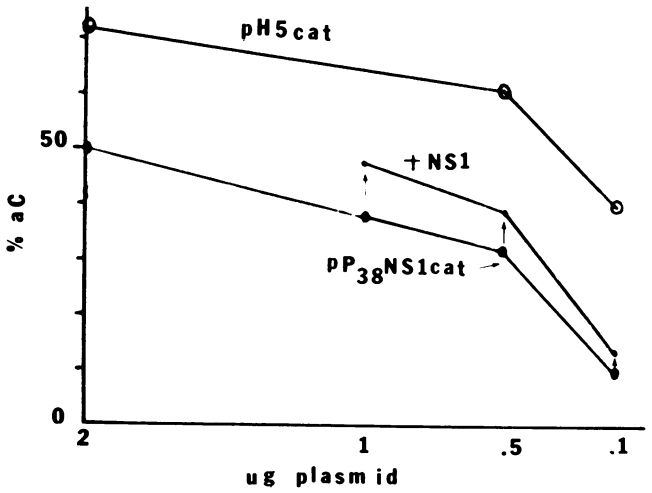

FIG. 2. Comparison of pH5cat and pP38NS1cat. The yield of *cat* activity of NB cells after transfection with various amounts of each plasmid are expressed as the percentage of chloramphenicol acetylated (aC). For the pP38NS1cat plasmid, replicate plates were also cotransfected with 2 μg of pH5, which expresses NS1 from the P4 promoter (shown as +NS1).

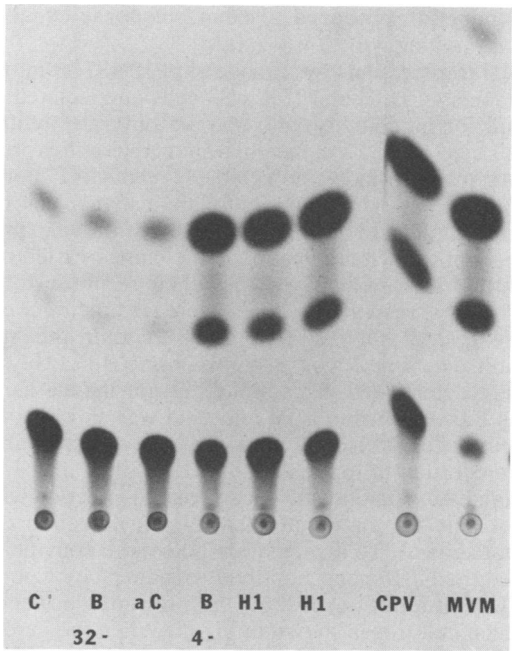

FIG. 3. Autoradiogram of CAT assay of the cell line NBA6. Dishes (60 mm) of NBA6 (3×10^5 cells per dish) were treated as follows: C, control, no treatment; B, 32−, 5 mM sodium butyrate from 32 to 48 h; aC, 5 mM azacytidine from 0 to 24 h; B, 4−, 5 mM sodium butyrate from 4 to 24 h; H1, left, superinfected with H-1 at 28 h; H1, right, superinfected with H-1 at 24 h; CPV, superinfected with canine parvovirus at 28 h; and MVM, superinfected with minute virus of mice at 28 h. The viruses were used at a multiplicity of infection of 10 to 20. Cell extracts were made at 48 h, and CAT activity was determined.

tk^+ colonies. A sampling of eight clones transfected with ptk26 and pH3cat in experiment 1 were grown to mass culture, and five of the eight had detectable CAT activity. Three colonies resulting from the cotransfection of *tk* and pH5cat had no detectable CAT activity. We also constructed a plasmid, pH5tk, in which the *tk* gene was positioned to be *trans*-activated by NS1, and this construction was very inefficient at producing tk^+ colonies (data not shown). Assay of Rat 2 cells 48 h after transfection with pH5tk verified that they produced thymidine kinase (data not shown). In all these results, whether the NS1-NS2 gene was in *trans* or in *cis* to the selectable marker gene, NS1-NS2 reduced the yield of stable transformants after transfection with a number of dominant selectable marker genes, suggesting that this gene has a toxic effect.

Transient expression of pP38NS1cat. To circumvent the apparent toxicity of P4-driven NS1-NS2, I constructed the plasmid pP38NS1cat (Fig. 1). This construct should place NS1-NS2 under the low constitutive expression of P38; but in the presence of threshold amounts of NS1, the *trans*-activation of the P38 promoters will provide an autostimulatory expression of NS1-NS2 as well as the *cat* gene. In transient assays in NB cells, pP38NS1cat showed a rather high level of expression, although not as high as that of pH5cat (Fig. 2). As a test for the presence of competent cells that had taken up pP38NS1cat but had not expressed it, additional NS1 was made available to trigger the positive feedback loop of pP38NS1cat by cotransfection with pH5, a plasmid that expressed NS1 under the P4 promoter. There was some increase in *cat* expression in the presence of pH5 (Fig. 2).

Stable cell lines containing pP38NS1cat. The NB cell line that is permissive for H-1 infection and is used for plaque titrations was cotransfected with 1 μg of pSV2neo and 4.5 μg of pP38NS1cat per 60-mm dish. The yield of colonies was 12 and 13 for two such dishes as compared with 31 colonies in a dish transfected with pSV2neo in the absence of pP38NS1cat. Five G418-resistant colonies were grown in mass culture and tested for *cat* expression with and without infection with H-1 20 h before the cell extraction. Four of the five clones had some expression of *cat*; two clones had very low levels and were not inducible on superinfection with virus, and two had at least a 10-fold increase in *cat* expression with infection. One of these, NBA6, was chosen for further study. The cell line was screened with various treatments to determine what could induce an increased *cat* expression. Infection with the parvoviruses H-1, minute virus of mice, and canine parvovirus induced strong responses (Fig. 3). Treatment of the cultures with 5-azacytidine at 5 μM for 24 h followed by a 24-h chase failed to stimulate *cat* expression. Sodium butyrate at 2.5 mM for 16 h followed by a chase of 24 h induced increased expression, while 16 h of sodium butyrate immediately before extraction was not effective.

The kinetics of the response were tested with replicate cultures treated with sodium butyrate and phorbol 12-tetradecanoate 13-acetate (TPA) and then chased for various times (Fig. 4). The cultures that were followed beyond 5 days were trypsinized at day 3, transferred to a T25 flask, and subcultured to a 60-mm dish 24 h before extraction. The final yield of enzyme was corrected for the cell dilution by the subculturing assuming a 100% plating efficiency. The induction of *cat* expression was slow, and very little increase was seen until about 36 h after the start of the induction. The levels of *cat* then rose rapidly to a peak at about day 3, and in this experiment the induced level reached 550-fold higher than the constitutive level. The yield of enzyme then gradually fell back to the constitutive level over 4 to 5 days. A

repeat induction produced a second response equal to the first (data not shown).

Similar transfections were made of pP38NS1cat into HeLa Gey monolayer cells and HeLa S3 cells and isolated clones screened for inducibility with sodium butyrate treatments. For HeLa S3, 4 of 12 clones had inducible *cat* expression, and the remaining had no detectable level of CAT. For HeLa Gey, four of six clones had inducible enzyme, one had no detectable CAT, and one had a weak constitutive expression that was not inducible. The inducible clones varied in their constitutive levels of enzyme and their response to induction. The most responsive clones increased their *cat* expression by greater than 20-fold at 48 h after the start of induction. The kinetics of response of one HeLa Gey clone, G3, were determined. This clone did not induce as high a level as NBA6, reaching 100-fold, and was slower to return to baseline. Otherwise the response was very similar.

Demonstration of induction of NS1. Pooled anti-H-1 antisera from hamsters that survived neonatal infection with H-1 was not efficient for immunofluorescent detection of NS1 (data not shown). To demonstrate NS1 more convincingly, I obtained the NS1-specific antibody produced by Cotmore et al. (5). Immunofluorescent staining of induced and uninduced cultures is shown in Fig. 5. When the percentage of positive cells was counted, it was found that positive cells were very rare (<1 in 1,000) on the noninduced control, with only five found on the whole cover slip at 48 h. The induced cultures reached an apparent high of 10% positive cells for NS1 at 48 h postinduction. In another experiment, a high of 20% was reached. The induced cultures showed some toxicity to the induction in this experiment, and some cells were lost from the cover slip. It is not known whether the cells that were lost were induced to express NS1 or not. The 100- to 500-fold increase in *cat* activity with induction seems to correlate roughly with the increase in cells staining positively for NS1. This result suggests that the bulk of the constitutive level of *cat* expression can be accounted for by a low number of cells that spontaneously induce high levels of NS1 expression. In the H-1-infected cells that were lightly stained for NS1, there was an apparent pattern of localization visible within the nucleus superimposed on a more generalized lightly stained background (Fig. 5D).

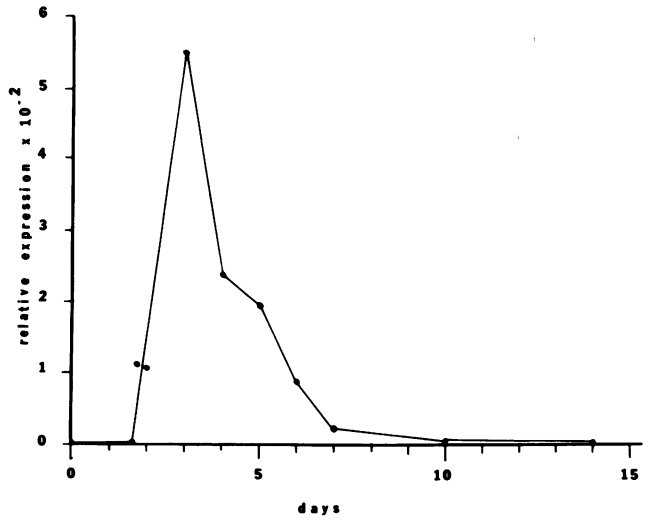

FIG. 4. Kinetics of *cat* expression in NBA6 induced with sodium butyrate and TPA. Replicate dishes of NBA6 were treated with 5 mM sodium butyrate and TPA at 0.1 ng/ml for 24 h. The cultures were collected for determination of CAT at the times indicated. After 5 days the cultures were subcultured, and the yield of enzyme was corrected for the dilution of the cells.

Inducible expression of H-1 coat protein gene. As a test of the capacity of the inducible NS1-NS2 cassette to *trans*-activate additional genes directed by a P38 promoter and integrated by a separate transfection, I produced clones of NBA6 transfected with p2VP. This plasmid has a tandem duplication of the P38 promoter that displays a moderate constitutive expression level and a strong response to *trans*-activation by NS1 when fused to the *cat* gene (S. Rhode and S. Richard, submitted for publication). In this construct the promoters are fused to the coat protein gene of the H-1 temperature-sensitive mutant *ts*6, which was subcloned from the genomic clone pSR1 (21). In transient expression assays examined by immunofluorescence, the plasmid produced globular nuclear antigen with an appearance identical to that after infection with the mutants *ts*1 (22) or *ts*6. The stable transformants were produced by cotransfection of NBA6 with pSV2gpt and p2VP. When cultures were treated with 1 μg of pSV2gpt and 10 μg of p2VP, the yield of colonies was reduced by 70%, and 0.5 μg of pSV2gpt and 15 μg of p2VP gave no colonies. A total of eight clones were expanded to mass culture and tested by immunofluorescence for H-1 antigens after induction with sodium butyrate. Two clones showed positive nuclei with the characteristic pattern of *ts*6, and three showed rare cells with a *ts*6 pattern of nuclei. One clone, NBA6(p2VP1), was selected for further study. Figure 6 illustrates the immunofluorescence staining of NBA6(p2VP1) with and without the sodium butyrate induction. The percentage of positive cells was low, about 10% at 48 h after induction. The appearance of the cells suggests that the level of antigen produced in the positive cells approaches that produced by infection with *ts*6. This result establishes that additional genes, other than *cat*, which are driven by the P38 promoter can be introduced as stable integrates into cells with an inducible *trans*-activation of expression and be coordinately regulated by NS1.

Improvement in induction efficiency. To maximize the capability of this inducible expression system to produce proteins from cloned genes, the efficiency of induction must be increased from the current levels of about 10 to 20%. One approach is to introduce the NS1 gene into cells with two types of inducible promoters, one that responds to a direct signal and a second which responds to the autostimulatory NS1 induction (i.e., pP38NS1cat). This was tested with a plasmid that used the mouse mammary tumor virus promoter, enhanced by the Harvey sarcoma enhancer on the 5′ side of the mouse mammary tumor virus promoter as in the plasmid pM14-1 described by Ostrowski et al. (16), to produce NS1 in response to dexamethasone. This plasmid, pHMNS1cat (Fig. 1), had a low level of *cat* expression in transient assays (0% acetylated) and responded well to dexamethasone stimulation (48% acetylated). When stable clones of NBA6 with pHMNS1cat or HeLa S3 cells with pP38NS1cat and pHMNS1cat were isolated and tested, only modest responses were obtained with dexamethasone, and they were of lower magnitude than the responses to butyrate (data not shown). These results suggest that in most of the cells with the integrated pP38NS1cat, the promoters are not in a configuration that responds well in *trans* to NS1. This state is altered by butyrate in a proportion of the cells. If the efficiency of induction is to be increased, a new approach to making the *trans*-activation-responsive element (*tar*) element more accessible to NS1 is needed.

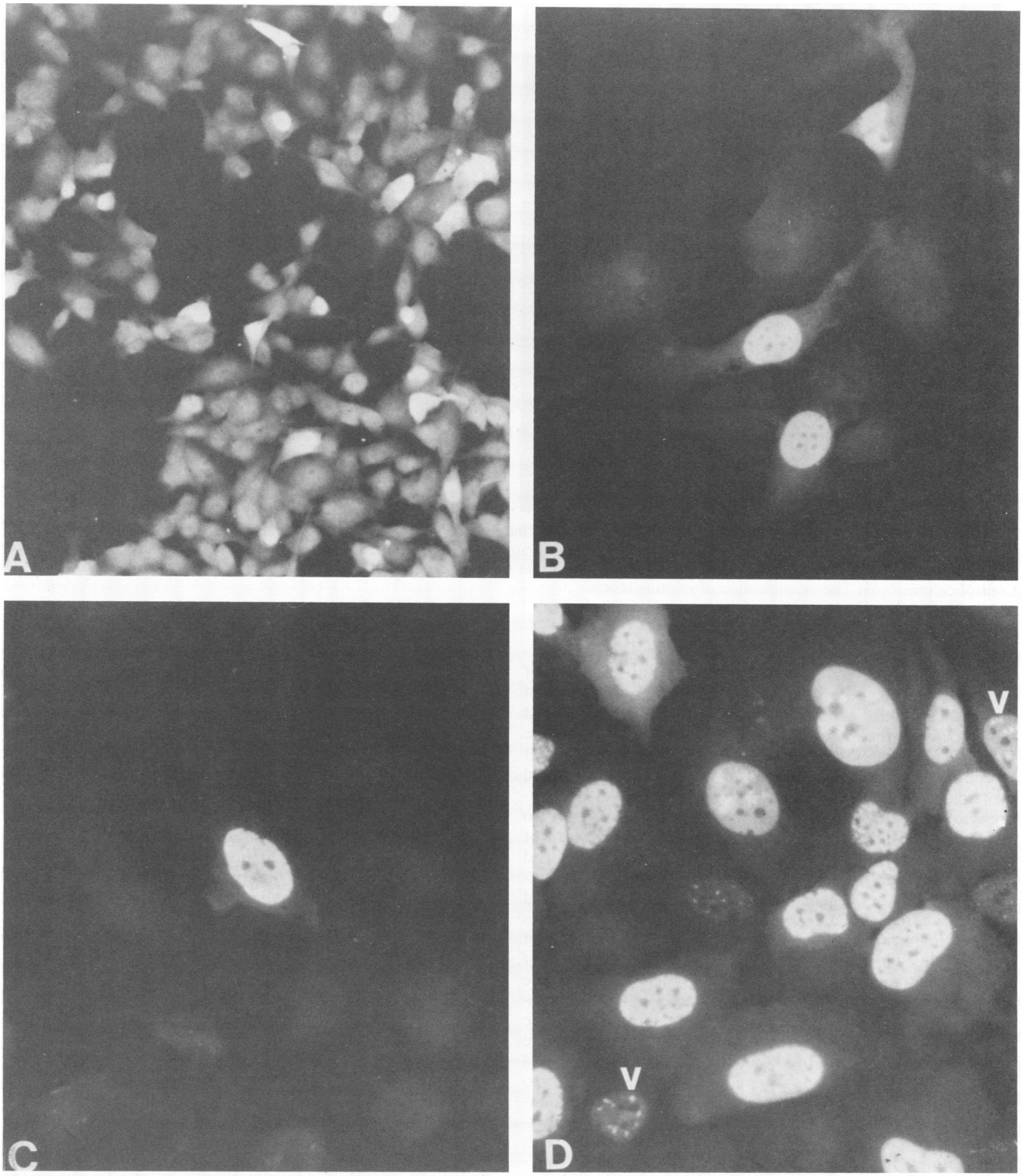

FIG. 5. Immunofluorescent micrographs of NBA6 cells stained with rabbit anti-NS1. The cultures of NBA6 were induced with sodium butyrate and fixed and stained with the NS1-specific antibody at 48 h postinduction. (A) Lower magnification (×25) of NS1-positive cells in induced culture. (B) Induced cells. (C) NS1-positive cell in a noninduced control culture. (D) NB cells 20 h postinfection with H-1 showing the focal concentrations of NS1 (open arrowheads). B, C, and D are at ×100. The magnification of printing is ×5.

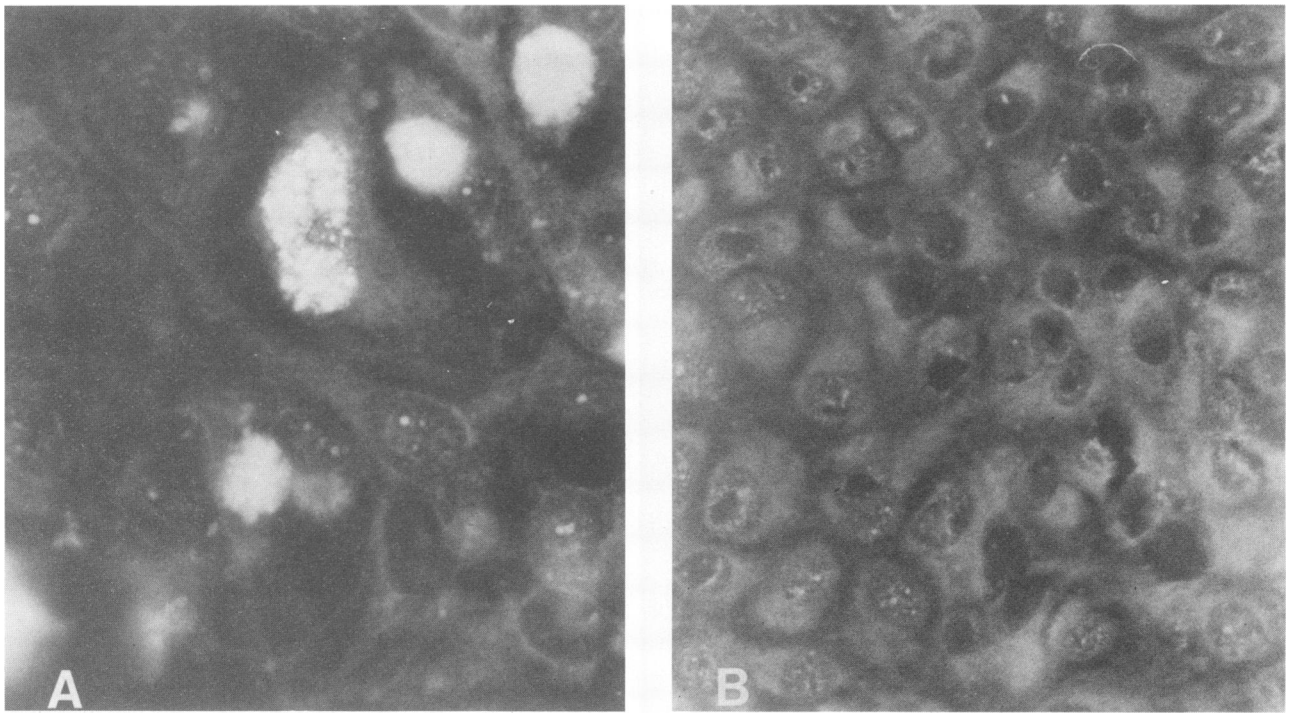

FIG. 6. Immunofluorescent micrographs of NBA6(p2VP) cells induced or not induced with sodium butyrate and stained with anti-H-1 antibody. The NBA6(p2VP) cells were treated with sodium butyrate and TPA and fixed with acetone 48 h postinduction. The cells were stained with hamster anti-H-1 antibody obtained from hamsters that survived a neonatal infection with H-1. (A) Induced culture, ×100. (B) Control culture, ×63. The magnification of printing is ×5.

DISCUSSION

The autonomous parvoviruses are lytic, but the cause of death of the infected cell is not known. The amplification of viral DNA could be toxic, but it was previously observed that abortive infections of human diploid fibroblasts in which viral DNA replication is reduced by greater than 99% result in cell death (27). Similarly, the production of viral capsid proteins may be toxic, but when capsid proteins are expressed by a bovine papilloma vector the cells appear to survive (19). This could be the result of reduced synthesis of capsid protein in this situation or a lack of toxicity of capsid protein. Because I failed to obtain cell lines that constitutively expressed NS1 when the NS1 gene was cotransfected into cells with a selectable marker, I established cell lines stably transformed with the plasmid pP38NS1cat. In some of the cell lines with integrated pP38NS1cat, elevated levels of *cat* were induced by superinfection. When the inducible clones were treated with sodium butyrate, sodium propionate, or TPA they showed induction of *cat* expression. The treatment that was near optimal was 5 mM sodium butyrate and 0.1 ng of TPA for 18 to 24 h, followed by a chase of 24 to 48 h. After reaching a peak level of *cat* expression in 48 to 72 h postinduction, the cultures gradually returned to constitutive levels of *cat* expression. In transient assays, treatment with sodium butyrate after transfection with pP38NS1cat also stimulated expression of the *trans*-activated *cat* gene, as found previously for certain enhancer-driven expression plasmids (6). Whether this is the result of alterations in chromatin assembly on the input DNA because of histone hyperacetylation or some other mechanism is not clear (3, 20, 24).

The availability of an NS1-specific antiserum permitted me to confirm that induction was in fact accompanied by the induction of expression of NS1 and that the level of cells responding was about 10 to 20%. The decline in *cat* expression, even though the design of the expression system would predict it to be locked on, is compatible with the hypothesis that NS1 expression is toxic. No direct demonstration of cell death as a result of NS1 expression has been obtained to date. NS1 has been shown to have a potent inhibitory effect on the expression of heterologous promoters in transient assays, and it inhibits cell DNA synthesis (S. Rhode, unpublished data). Although NS1 may inhibit the expression of a selectable marker when it contains a heterologous promoter, the low yield of transformants with pH5tk or pH5neo, in which the expression of the marker gene is NS1 dependent, suggests that this is not the only reason for the inhibition of transformation.

It is not clear whether cell lines such as NBA6 or NBA6(p2VP1) can be used to propagate viral mutants. I showed here that the coat protein gene can be stably integrated into an inducible cell line with a separate transfection and that it will respond to *trans*-activation by NS1. Thus, it is possible to configure a cell line that would provide capsid proteins for $NS1^+$, capsid protein mutant viruses. I am currently testing NBA6 for its ability to propagate an NS1 mutant.

A second consideration of importance in practical applications is the development of efficient means of inducing the expression system. The use of sodium butyrate and TPA to induce expression of the pP38NS1cat *trans*-activated loop was relatively inefficient with peak inductions in the range of 20%. Other genes that are inducible by sodium butyrate have been reported to respond at levels as high as 30% (26). The most common explanation for the induction with butyrate that has been proposed is that the hyperacetylation of histones that occurs as a result of butyrate inhibition of

histone deacetylase may alter the structure of chromatin to a more open configuration that favors the binding of transcription regulatory proteins (14). In this respect, butyrate may have an action related to the effects of TPA, as that compound has been reported to stimulate ADP ribosylation of histones (28). The reason that some clones express some *cat* constitutively but do not induce by superinfection or by butyrate is unknown.

The constitutive level of expression of NS1 was exceedingly low in NBA6, and it is unlikely that it would generate any selective pressure against the inducible phenotype of the culture. The NBA6 culture remained inducible after 40 passages in culture subsequent to its initial cloning. Thus, one can predict that if the constitutive level of expression with an inducible expression system is kept to a low level, then the cell lines should remain stable even when the proteins to be expressed are cytotoxic.

More efficient induction of the NS1 cassettes might be obtainable by coupling the NS1 gene to another inducible promoter and using this construct to provide threshhold levels of NS1 to induce the P38-driven NS1 gene(s). My initial attempts to achieve this used the glucocorticoid-responsive promoter of mouse mammary tumor virus and included the enhancer of Harvey sarcoma virus to increase the level of expression as reported by Ostrowski et al. (16). These experiments were not successful, as only a very low response was achieved with dexamethasone, and it was less than the butyrate response. This may imply that in most of the cells the P38 promoter is not in a state that will respond in *trans* to NS1 or that the dexamethasone response is not efficient after integration of the construct. Future attempts to increase the inducibility of this system will combine the hormone-inducible promoter with efforts to modify the chromatin structure of the P38 to a responsive configuration. The construction of genetic circuits with genes for *trans*-acting proteins and using novel configurations of *cis* elements regulating gene expression should lead to eucaryotic expression systems that will serve as useful tools in the sudy of cell behavior and for the production of proteins from cloned genes.

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LITERATURE CITED

1. **Astell, C. R., E. M. Gardiner, and P. Tattersall.** 1986. DNA sequence of the lymphotropic variant of minute virus of mice, MVM(i), and comparison with the DNA sequence of the fibrotropic prototype strain. J. Virol. **57**:656–669.
2. **Astell, C. R., M. Thomson, M. Merchlinsky, and D. C. Ward.** 1983. The complete DNA sequence of minute virus of mice, an autonomous parvovirus. Nucleic Acids Res. **11**:999–1018.
3. **Candido, E. P. M., R. Reeves, and J. R. Davie.** 1978. Sodium butyrate inhibits histone deacetylation in cultured cells. Cell **14**:105–113.
4. **Cotmore, S., and P. Tattersall.** 1986. Organization of nonstructural genes of the autonomous parvovirus minute virus of mice. J. Virol. **58**:724–732.
5. **Cotmore, S. F., L. J. Sturzenbecker, and P. Tattersall.** 1983. The autonomous parvovirus MVM encodes two non-structural proteins in addition to its capsid polypeptides. Virology **129**:333–343.
6. **Gorman, C. M., and B. H. Howard.** 1983. Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate. Nucleic Acids Res. **11**:7631–7648.
7. **Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn.** 1977. Characterization of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36**:59–72.
8. **Jongeneel, C. V., R. Sahli, G. K. McMaster, and B. Hirt.** 1986. A precise map of splice junctions in the mRNAs of minute virus of mice, an autonomous parvovirus. J. Virol. **60**:564–573.
9. **Keller, J. M., and J. C. Alwine.** 1984. Activation of the SV40 late promoter: direct effects of T antigen in the absence of viral DNA replication. Cell **36**:381–389.
10. **Labow, M. A., P. L. Hermonat, and K. I. Berns.** 1986. Positive and negative autoregulation of the adeno-associated virus type 2 genome. J. Virol. **60**:251–258.
11. **Lebovitz, R. M., and R. G. Roeder.** 1986. Parvovirus H-1 expression: mapping of the abundant cytoplasmic transcripts and identification of the promoter sites and overlapping transcription units. J. Virol. **58**:271–280.
12. **Molitor, T. W., H. S. Joo, and M. S. Collett.** 1985. Identification and characterization of a porcine parvovirus nonstructural polypeptide. J. Virol. **55**:554–559.
13. **Mulligan, R. C., and P. Berg.** 1981. Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase. Proc. Natl. Acad. Sci. USA **78**:2072–2076.
14. **Nelson, D. A., R. C. Ferris, D. Zhang, and C. R. Ferenz.** 1986. The beta-globin domain in immature chicken erythrocytes: enhanced solubility is coincident with histone hyperacetylation. Nucleic Acids Res. **14**:1667–1682.
15. **Nevins, J. R.** 1981. Mechanism of activation of early viral transcription by the adenovirus E1A gene product. Cell **26**:213–220.
16. **Ostrowski, M. C., A. L. Huang, M. Kessel, R. G. Wolford, and G. L. Hager.** 1984. Modulation of enhancer activity by the hormone responsive regulatory element from mouse mammary tumor virus. EMBO J. **3**:1891–1899.
17. **Paradiso, P. R.** 1984. Identification of multiple forms of the noncapsid parvovirus protein NCVP1 in H-1 parvovirus-infected cells. J. Virol. **52**:82–87.
18. **Paradiso, P. R., K. R. Williams, and R. L. Costantino.** 1984. Mapping of the amino terminus of the H-1 parvovirus major capsid protein. J. Virol. **52**:77–81.
19. **Pintel, D., M. J. Merchlinsky, and D. C. Ward.** 1984. Expression of minute virus of mice structural proteins in murine cell lines transformed by bovine papillomavirus-minute virus of mice plasmid chimera. J. Virol. **52**:320–327.
20. **Reeves, R., C. M. Gorman, and B. Howard.** 1985. Minichromosome assembly of non-integrated plasmid DNA transfected into mammalian cells. Nucleic Acids Res. **13**:3599–3615.
21. **Rhode, S. L., III.** 1985. *Trans*-activation of parvovirus P_{38} promoter by the 76K noncapsid protein. J. Virol. **55**:886–889.
22. **Rhode, S. L., III.** 1976. Replication process of the parvovirus H-1. V. Isolation and characterization of temperature-sensitive H-1 mutants defective in progeny DNA synthesis. J. Virol. **17**:659–667.
23. **Rhode, S. L., III, and P. R. Paradiso.** 1983. Parvovirus genome: nucleotide sequence of H-1 and mapping of its genes by hybrid-arrested translation. J. Virol. **45**:173–184.
24. **Sahli, R., G. K. McMaster, and B. Hirt.** 1985. DNA sequence comparison between two tissue-specific variants of the autonomous parvovirus, minute virus of mice. Nucleic Acids Res. **13**:3617–3633.
25. **Sealy, L., and R. Chalkley.** 1978. The effect of sodium butyrate on histone modification. Cell **14**:115–121.
26. **Shein, H. M., and J. F. Enders.** 1962. Multiplication and cytopathogenicity of simian vacuolating virus 40 in cultures of human tissues. Proc. Soc. Exp. Biol. Med. **109**:495–500.
27. **Singer, I. I., and S. L. Rhode III.** 1978. Electron microscopy and cytochemistry of H-1 parvovirus intracellular morphogenesis, p. 479–504. In D. C. Ward and P. Tattersall (ed.), Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

28. **Singh, N., and P. Cerutti.** 1985. Poly ADP-ribosylation of histones in tumor promoter phorbol-12-myristate-13-acetate treated mouse embryo fibroblasts C3H10T1/2. Biochem. Biophys. Res. Commun. **132**:811–819.
29. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
30. **Topp, W. C.** 1981. Normal rat cell lines deficient in nuclear thymidine kinase. Virology **113**:408–411.
31. **Tratschin, J. D., J. Tal, and B. J. Carter.** 1986. Negative and positive regulation in *trans* of gene expression from adeno-associated virus vectors in mammalian cells by a viral *rep* gene product. Mol. Cell. Biol. **6**:2884–2894.