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Inducible Expression of the P, V, and NP Genes of the Paramyxovirus Simian Virus 5 in Cell Lines and an Examination of NP-P and NP-V Interactions

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The P, V, and NP genes of the paramyxovirus simian virus 5 (SV5) were cloned such that their expression was regulated by the tetracycline-controlled transactivator (M. Gossen and H. Bujard, Proc. Natl. Acad. Sci. USA 89:5547–5551, 1992), and mammalian cell lines that inducibly expressed individually the P, V, or NP protein or coexpressed the P plus NP or V plus NP proteins were isolated. A plasmid that expresses the tetracycline-controlled transactivator linked, via the foot-and-mouth disease virus 2A cleavage peptide sequence, to the neomycin aminoglycoside phosphotransferase gene was constructed. Cells were cotransfected with this plasmid, and the appropriate responder plasmids and colonies were selected on the basis of their resistance to Geneticin (via the neomycin aminoglycoside phosphotransferase gene). The properties of these cell lines, in terms of the induction of the P, V, and NP genes, are described in detail. Both the P and V proteins were phosphorylated when expressed alone. In immunoprecipitation studies using a monoclonal antibody that recognizes both the P and V proteins, a nonphosphorylated host cell protein with an estimated molecular weight of 150,000 was coprecipitated with V but not P. Immunofluorescence data demonstrated that when expressed separately, the P protein had a diffuse cytoplasmic distribution, but the related V protein had both a nuclear and cytoplasmic distribution. The NP protein had a granular cytoplasmic distribution, giving rise to punctate and granular fluorescence. Coexpression of the NP and P proteins resulted in the accumulation of large cytoplasmic inclusion aggregates, similar to those visualized at late times in SV5-infected cells. Coexpression of V with NP led to a partial redistribution of the NP protein in that the NP protein had both a diffuse cytoplasmic and nuclear distribution in the presence of V, but no NP-V aggregates or inclusion bodies were visualized. Direct binding studies also revealed that NP bound to both P and V. For SV5, these studies suggest that V may have a role in keeping NP soluble prior to encapsidation.

Simian virus 5 (SV5), like other paramyxoviruses, has a nonsegmented negative-stranded RNA genome found in a helical nucleocapsid, composed primarily of the nucleocapsid protein NP, which is surrounded by a viral envelope (for a review, see reference 13). Associated with the nucleocapsid are the polymerase protein (L), the phosphoprotein (P), and the V protein (26). The P and V proteins are amino coterminal but differ in their C-terminal sequences (33). The C terminus of V is cysteine rich and is highly conserved between different paramyxoviruses. The V proteins of measles virus and SV5 have been reported to bind zinc (22, 26). Encapsidation of paramyxovirus RNA occurs during RNA replication and is thought to be a tightly controlled process requiring NP-P and P-L protein-protein interactions (6, 17, 19, 29). In addition to these proteins, the V protein may also be involved in the control of virus transcription and replication. It has been shown in the Sendai virus system that V (and a related protein, W) can down-regulate virus replication but not transcription (4, 6). Furthermore, it has also been suggested, but not shown, that V proteins may interact weakly with the NP and L proteins (5). Here, we demonstrate that V can interact with NP and that this binding can be inhibited by the presence of excess P but not vice versa.

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The NP, P, and L proteins of SV5 have been observed to colocalize in virus-infected cells, often in large cytoplasmic inclusion bodies (10, 11). We have suggested that paramyxoviruses may remain quiescent in such inclusion bodies for prolonged periods of time and that cells harboring inclusion bodies (but not actively synthesizing virus proteins) may remain hidden from cell-mediated immune responses (11). To begin to elucidate further the control of paramyxovirus transcription and replication and the role of inclusion bodies in paramyxovirus infections, we wished to develop systems to examine protein-protein interactions in cells in a controlled manner.

The system that we have been exploring for these purposes is the inducible system for expressing foreign gene products in mammalian cells that was developed by Gossen and Bujard (16). This system is based on regulatory elements of the tetracycline resistance operon of *Escherichia coli* (18). In *E. coli*, tetracycline resistance gene expression is regulated by the tetracycline repressor (*tetR*). In the absence of tetracycline, *tetR* binds to the promoter and down-regulates expression; in the presence of tetracycline, *tetR* does not bind to the promoter, thus allowing transcription of the operon. To adapt this system for inducible expression in mammalian cells, a chimeric tetracycline-controlled transcriptional activator protein, termed tTA, has been made between the *E. coli* Tn*10*-encoded *tetR* and the transcriptional activation domain of VP16 of herpes simplex virus. Genes of interest are cloned downstream of a heptamerized *tet* operator and human cytomegalovirus minimal promoter, and expression is regulated by the presence (no

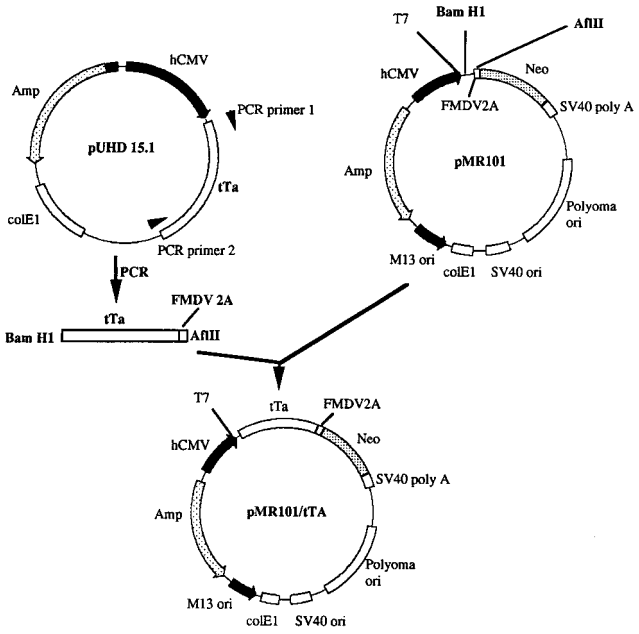

FIG. 1. Construction of plasmid pMR101/tTa. PCR primers were constructed to amplify the tTa gene from pUHD 15-1. Primer 1 had a site for the restriction enzyme *Bam*HI 5′ to the start of the coding region of tTa. Primer 2 contained sequences encoding the FMDV 2A gene (including an *Afl*II restriction site) downstream of the 3′-terminal tTa sequences (which excluded the stop codon). The PCR product was cloned into plasmid pMR101, and in the resulting construct (pMR101/tTA), tTa, FMDV 2A, and Neo formed a single open reading frame. Also shown on the plasmid maps are the positions for human cytomegalovirus (hCMV) and T7 promoters, the simian virus 40 (SV40) poly(A) addition site, polyomavirus and simian virus 40 origins of replication (ori), *E. coli* (colE1) and M13 origins of replication, and a gene encoding ampicillin resistance (Amp).

expression, as opposed to *tetR* in *E. coli*) or absence (expression) of tetracycline (15, 16).

To establish cell lines that constitutively express tTA, plasmids encoding this protein are usually cotransfected into cells with a separate plasmid which confers a resistance phenotype such as neomycin aminoglycoside phosphotransferase (Neo) resistance, which results in resistance to the neomycin analog Geneticin) (15). Alternatively, a gene which specifies a resistance phenotype is cloned into the same plasmid as the plasmid specifying the tTA protein. However, the level of success with these approaches is variable, and many cell lines may have to be screened before one which constitutively expresses sufficiently high levels of tTA to be useful is obtained. Here, we describe an alternative approach in which the gene encoding tTA has been fused, via an intervening sequence which encodes the foot-and-mouth disease virus (FMDV) 2A cleavage peptide sequence (30), to the Neo gene in a single long open reading frame. We show that cleavage of the fusion protein occurs at the 2A site to produce fully functional tTA and Neo. Using this approach, we have isolated cell lines which inducibly express the P, V, and NP proteins of the paramyxovirus SV5 and examine have the nature of NP-P and NP-V interactions.

MATERIALS AND METHODS

Plasmids and general recombinant DNA protocols. The driver plasmid, encoding tTA, pUHD 15-1, the response plasmid pUHD 10-3, and the luciferase reporter plasmid pUHD 13-3 have been described in detail elsewhere (15, 16). Plasmid pMR101, which encodes the FMDV 2A protease cleavage site in frame with the Neo gene, will be described in detail elsewhere (9), but its general characteristics are described in Fig. 1.

Plasmid DNAs were isolated by using the alkaline lysis method of Birnboim and Doly (1a) and treated by standard protocols (23). Enzymes used for recombinant DNA work were purchased from New England Biolabs, Inc. (Bishop's Stortford, Hertfordshire, England) or Bethesda Research Laboratories (Paisley, Scotland) and used as recommended by the manufacturer. *E. coli* Top10 was made competent by using the $CaCl_2$ method described by Maniatis et al. (23).

Generation of pMR101/tTA pUHD 10-3/SV5/P, pUHD 10-3/SV5/V, and pUHD 10-3/SV5/NP. Oligonucleotides used in this work were synthesized by using an Applied Biosystems oligonucleotide synthesizer and phosphoramidite chemistry. The sequences of the forward and reverse primers for amplification of the tTA gene were 5′ AAATTTggatccGCCGCCACCATGTCTAGATTAGATAAAAG TAAAG and 5′ GCAAGcttaag*AAGGTCAAAATTCAACAGCTG*CCCACCGT ACTCGTCAATTC, respectively (nucleotides in lowercase indicate the restriction enzyme sites, underlined nucleotides are complementary to the tTA gene, nucleotides in italics represent sequences from FMDV 2A).

These primers were used to amplify the tTA gene from pUHD 15-1. The resulting 1.2-kb PCR product was separated on an agarose gel, excised, and purified on a Spin Bind column (Flowgen, Sittingbourne, Kent, England). The fragment was then cut with restriction enzymes *Bam*HI and *Afl*II and ligated into pMR101 that had been digested with the same restriction enzymes and dephosphorylated. The ligated product was transformed into competent *E. coli* Top 10 cells. Colonies were screened for the presence of the tTA gene by using as a probe the tTA PCR product that had been radiolabelled with ^{32}P by random priming. Positive colonies were selected and grown, and the DNA was isolated.

Briefly, to construct plasmids pUHD 10-3/SV5/P, pUHD 10-3/SV5/V, and pUHD 10-3/SV5/NP, the P, V, and NP genes were amplified from SV5 (strain W3a)-infected cells by reverse transcription and PCR, and the PCR products were first cloned into pGEM3 (Promega, Madison, Wis.). The genes were then directly subcloned from the pGEM3 vectors into the tTA responder plasmid pUHD 10-3 (between the *Eco*RI and *Bam*HI sites), resulting in the generation of plasmids pUHD 10-3SV5/P, pUHD 10-3SV5/V, and pUHD 10/3SV5/NP. It should also be noted that in plasmid pUHD 10-3/SV5/NP, the rabbit β-globin leader sequence (20) was added prior to the translation start site, i.e., ACACTT GCTTTTGACACAACTGTGTTTACTTGCAATCCCCCAAAACAGACAG A*ATG* (start codon in italics).

Cells, transfection protocols, and isolation of cell lines. BALB/c fibroblast (BF), baby hamster kidney (BHK), and Vero cells were grown as monolayers in 25- or 75-cm^2 flasks in Dulbecco's modification of Eagle's tissue culture medium supplemented with 10% newborn calf serum and were negative for mycoplasmas as screened by 4′, 6-diamidino-2-phenylindole (DAPI) staining. In transient transfection assays (used for measuring activation of a reporter gene, the luciferase gene, by tTA), Vero cells in 35-mm^2 tissue culture petri dishes were transfected with 5 μg of the luciferase reporter plasmid pUHD 13-3 and 5 μg of either pMR101/tTA or pUHD 15-1, using TransfectACE (Gibco-BRL) as recommended by the manufacturer for 18 to 24 h. The TransfectACE was removed, the cells were reincubated in culture medium for a further 36 to 48 h and harvested, and luciferase activity was measured as described below.

To isolate cell lines that permanently expressed the P, V, and/or NP proteins of SV5, confluent monolayers of BF cells in 75-cm^2 flasks were cotransfected with (i) pMR101/tTA and pUHD 10-3SV5/P, (ii) pMR101/tTA and pUHD 10-3SV5/NP, (iii) pMR101/tTA and pUHD 10-3SV5/V, and (iv) pMR101/tTA and pUHD 10-3SV5/P plus pUHD 10-3SV5/NP, at a 1:10 ratio of driver plasmid to responder plasmid (40 μg of driver plasmid and 400 μg of responder plasmid per 75-cm^2 flask). At 24 h after the addition of the TransfectACE DNA mixtures, the cells were trypsinized and reseeded into two 150-mm-diameter tissue culture petri dishes. After a further 24 h, Geneticin (400 μg/ml) was added to the tissue culture medium. At all times, the cells were also grown in the presence of tetracycline (2 μg/ml). When colonies of cells had grown sufficiently large to be visualized by eye, individual colonies were ring cloned and grown in 24-well Linbro plates and screened for expression by immunofluorescence as described below. Selected colonies were amplified and grown in 25- or 75-cm^2 tissue culture flasks. To induce expression of the tTA-responsive genes, the cell monolayers were washed four times with tissue culture medium without tetracycline or Geneticin and reincubated in the same medium for various periods of time as described in Results.

Luciferase assays. Transfected cells were washed twice with ice-cold phosphate-buffered saline (PBS). To each 35-mm-diameter petri dish, 200 μl of luciferase lysis buffer (25 mM Tris-HCl [pH 7.5], 8 mM $MgCl_2$, 1 mM dithiothreitol, 1% Triton X-100, 15% glycerol) was added, and the plates were kept on ice for 5 min. The cells were scraped into the lysis buffer, and the lysate was transferred to Eppendorf tubes. Then 100 μl of luciferase buffer (1 mM ATP, 250 μM Luciferin, 1% bovine serum albumin) and lysate were mixed in a cuvette, and luciferase activity was measured in a luminometer (BioOrbit) as instructed by the manufacturer.

Antibodies. A detailed description of the monoclonal antibodies (MAbs) to the SV5 NP and P proteins and their nomenclature has been given elsewhere (28). The antibody used to detect the P and V protein by Western blotting (immunoblotting), immunoprecipitation, and immunofluorescence was termed SV5-Pk and reacts with a short nine-amino-acid epitope located on the N-terminal domain of both proteins (31). The antibody SV5-NPa was used to detect the NP protein by immunoprecipitation and immunofluorescence, but the antibody SV5-NPd was used in Western blotting to detect the NP protein (27).

Rabbit anti-neomycin phosphotransferase II was purchased from 5′ Prime → 3′ Prime Inc., Colo.) The rabbit antiserum used to detect tTA was raised against the acidic domain of herpes simplex virus VP16 and was a kind gift from P. O'Hare (Marie Curie Memorial Foundation Research Institute, Oxted, Surrey, England).

Preparation of radiolabelled antigen extracts, immunoprecipitation, and SDS-polyacrylamide gel electrophoresis. BF cell monolayers in 25-cm^2 tissue culture flasks were radioactively labelled for 2 h with either L-[^{35}S]methionine (10 μCi/ml; >1,000 Ci/mmol; Amersham International Ltd.) in tissue culture medium containing 1/10 the normal concentration of methionine (i.e., 1.5 mg/liter) or [^{32}P]phosphate (300 μCi/ml; Amersham International Ltd.) in phosphate-free medium plus 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.0). At the end of the labelling interval, the cells were washed in ice-cold PBS and lysed into immunoprecipitation buffer (10 mM Tris-HCl [pH 7.2], 5 mM EDTA, 0.5% Nonidet P-40, 0.65 M NaCl, 0.1% sodium dodecyl sulfate [SDS]; 4×10^6 to 6×10^6 cells per ml of buffer) by sonication with an ultrasonic probe. Soluble antigen extracts were obtained after pelleting of the particulate material from the total cell antigen extracts by centrifugation at 400,000 × *g* for 30 min. Immune complexes were formed by incubating 1-ml samples of the soluble antigen extracts with an excess of antibody for 2 h at 4°C. The immune complexes were isolated (21) on an excess of a fixed suspension of the Cowan A strain of *Staphylococcus aureus* (20 μl of a 10% [wt/vol] suspension per μl of ascitic fluid for 30 min at 4°C). The proteins in the immune complexes were dissociated by heating (100°C for 5 min) in gel electrophoresis sample buffer (0.05 M Tris-HCl [pH 7.0], 0.2% SDS, 5% 2-mercaptoethanol, 5% glycerol) and analyzed by electrophoresis through SDS–12% polyacrylamide gels. After electrophoresis, gels were dried and labelled polypeptides were visualized by autoradiography.

Western blot analysis. Samples to be analyzed were suspended in the appropriate volume of gel electrophoresis sample buffer, boiled for 5 min, and analyzed by electrophoresis through SDS–12% polyacrylamide gels. Separated polypeptides were transferred to nitrocellulose by using a semidry gel electroblotter (LKB). For Western blot analysis, any unoccupied protein binding sites on the nitrocellulose were blocked with 10% dried milk (Marvel)–0.1% polyoxyethylene sorbitan monolaurate (Tween 20) in PBS before the filters were incubated with the appropriate MAbs. Bound antibody was detected with horseradish peroxidase-conjugated protein A and enhanced chemiluminescence detection (Amersham International Ltd.).

Immunofluorescence. BF cells to be stained for immunofluorescence were grown on multispot microscope slides (C. A. Hendley Ltd., Essex, England) or 13-mm-diameter round coverslips (General Scientifics, Redhill, Surrey, England) in 24-well Linbro plates. The cells were treated and stained with specific MAbs as described in detail elsewhere (27). Briefly, monolayers were fixed with 5% formaldehyde–2% sucrose in PBS for 10 min at 20°C, permeabilized with 0.5% Nonidet P-40–10% sucrose in PBS for 5 min at 20°C, and washed three times in PBS containing 1% calf serum. For detection of the P protein, cells were stained by direct immunofluorescence using SV5-Pk that had been conjugated with fluorescein isothiocyanate (FITC); to detect the NP protein, cells were stained with MAb SV5-NPa that had been conjugated with rhodamine. In addition, cells were also stained with the DNA-binding fluorochrome DAPI. Following staining for immunofluorescence, the monolayers of cells were examined with either a Nikon Microphot-FXA immunofluorescence microscope or a Bio-Rad MRC600 confocal microscope.

Direct visualization of NP-P and NP-V interactions. Extracts of BF cells that had been induced for expression of the NP protein for 48 h were made by resuspending monolayer of cells, grown in a 75-cm^2 flask, in 1 ml of buffer (as detailed in Results). The cells were disrupted by sonication, insoluble material was pelleted by centrifugation at 13,000 rpm for 5 min in an MSE microcentrifuge, and the supernatant (soluble antigen) was collected. Cells expressing the P or V protein were grown on multispot microscope slides and, following induction of expression for 24 h, were fixed and permeabilized as described above for immunofluorescence. The slides were thoroughly washed with PBS to remove any traces of detergent. Soluble antigen extracts of the NP-expressing cells were then incubated with these fixed cells for 1 to 2 h at 4°C. The slides were washed four times with PBS and fixed with 5% formaldehyde–2% sucrose for 5 min. After the cells were washed four more times with PBS, the presence of the NP and P or V proteins was detected by immunofluorescence.

RESULTS

Expression of tTA-Neo. Plasmid pMR101/tTA, which encodes the tTA protein fused to Neo via the FMDV 2A cleavage site, was constructed as schematically detailed in Fig. 1. To determine whether cleavage of the tTA-2A-Neo protein occurred in tissue culture cells, we isolated cell lines that had been transfected with pMR101/tTA and had been selected on the basis of their resistance to Geneticin (below). These cells were analyzed for the presence of tTA and Neo by Western blot analysis. Figure 2 shows that proteins with the predicted molecular weights of the authentic tTA and Neo proteins could be detected but the precursor fusion protein could not, thus demonstrating the efficient cleavage of the fusion protein into the tTA and Neo proteins.

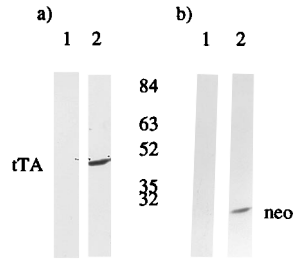

FIG. 2. Western blot analysis of total cell extracts of BF cells (lanes 1) and BF cells transfected with pMR101/tTA and selected on the basis of Neo resistance (lanes 2), using a polyclonal rabbit antiserum to tTA (a) or Neo (b). The positions of the molecular weight markers (indicated in kilodaltons) represents the positions to which they migrated on the gel.

Previous work (30) on fusion proteins containing the 2A cleavage domain has shown that cleavage at the 2A site results in the addition of 19 amino acids to the C terminus of the N-terminal domain (in this case the tTA protein) and a single amino acid (proline) to the N terminus of the C-terminal domain (in this case Neo). Since, cells that were resistant to Geneticin were isolated, it was clear that the addition of proline to the N-terminal end of Neo did not interfere with its function. However, it was important to determine whether the addition of 19 additional amino acids to the C-terminal end of tTA interfered with its ability to activate transcription. To examine this, we performed transient transfection assays in which plasmids pMR101/tTA and pUHD 15-1 were compared for the ability to activate a reporter plasmid, pUHD 13-3, that encodes luciferase under the control of a heptamerized *tet* promoter (16). These results clearly demonstrated that cotransfection of pUHD 13-3 with either pMR101/tTA or pUHD 15-1 resulted in similar levels of luciferase activity (Table 1).

Isolation of cell lines expressing P, V, NP, and NP plus P proteins of SV5. The P, V, and NP genes were first amplified from SV5-infected cells by reverse transcription and PCR, and the PCR product was cloned into pGEM3 (26a). The cloned products were sequenced by double-stranded DNA sequencing. Apart from two silent substitutions at positions 732 (numbered from the beginning of the open reading frame) and 1074 (both C-to-T transitions) in the NP gene, the sequence was identical to that published by Parks et al. (25). There were also two silent nucleotide substitutions (A to C and A to G, at positions 207 and 993, respectively) in the P gene compared

TABLE 1. Comparison of the abilities of the tTA driver plasmid, pUHD 15-1, and the tTA/neo driver plasmid, pMR101/tTA, to transactivate the luciferase reporter plasmid, pUHD 13-3, following transient cotransfection of 293 cells

Expt	Luciferase assay (RLU/10^6 cells)[a]	
	pUHD 15-1	pMR101/tTA
1	9,093	15,000
2	13,000	20,000
3	26,324	33,236
4	18,248	34,048

[a] The background level of luciferase activity observed after transfection of cells with the reporter plasmid in the absence of the driver plasmids was 20 to 100 relative luciferase units (RLU)/10^6 cells.

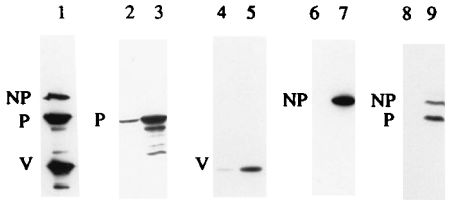

FIG. 3. Detection by Western blot analysis of the P, V, and NP proteins in total cell extracts of BF cells infected with SV5 (lane 1) or selected (by immunofluorescence) for the expression of the P (lanes 2 and 3), V (lanes 4 and 5), NP (lanes 6 and 7), and P plus NP (lanes 8 and 9) proteins. Cells were either grown continuously in the presence of tetracycline (lanes 2, 4, 6, and 8) or released from the tetracycline block for 24 h (lanes 3, 5, 7, and 9). Polypeptides were separated by electrophoresis through an SDS–12% polyacrylamide slab gel. The P and V proteins were detected with MAb SV5-Pk, and the NP protein was detected with MAb SV5-NPd.

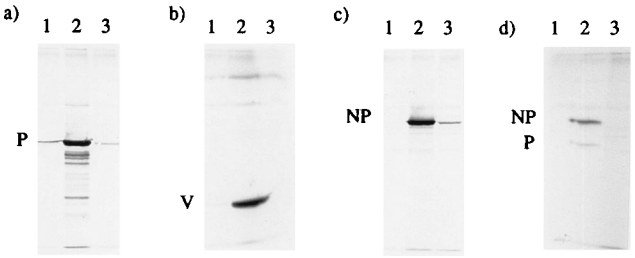

FIG. 4. Analysis of radioactively labelled polypeptides present in immunoprecipitates formed by the reaction of MAbs specific for P, V, and NP proteins with soluble antigen extracts made from BF cell lines expressing the P (a), V (b), NP (c), or P plus NP (d) proteins. Cells were grown in the continuous presence of tetracycline (lanes 1) or released from the tetracycline block for 24 h (lanes 2) before being recultured in the presence of tetracycline for 24 h (lanes 3). Cells were labelled with [^{35}S]methionine for 2 h prior to extraction. Polypeptides were separated by electrophoresis through an SDS–12% polyacrylamide slab gel and visualized by autoradiography. The extracts were precipitated with MAb SV5-Pk (a and b), MAb SV5-NPa (c) and both (d).

with the published sequence of Thomas et al. (33) (1). The P, V, and NP genes were then directly subcloned from the pGEM vectors into the tTA responder plasmid pUHD 10-3 (between the *Eco*RI and *Bam*HI sites), resulting in the generation of the responder plasmids pUHD 10-3SV5/P, pUHD 10-3SV5/V, and pUHD 10-3SV5/NP.

BF cells were cotransfected with pMR101/tTA together with the appropriate responder plasmids. We isolated 24 colonies from the cells transfected with pUHD 10-3SV5/NP, of which 11 were positive for NP; 17 colonies from cells transfected with pUHD 10-3SV5/P, of which 5 were positive for P; 15 colonies from cells transfected with pUHD 10-3SV5/V, of which 4 were positive for V; and 64 colonies from cells transfected with pUHD 10-3SV5/P and pUHD 10-3SV5/NP, of which 11 were clearly positive for both NP and P. Colonies which had the highest level of positive cells were subcloned, and the colonies were again screened for the presence of the P and/or NP proteins. One colony of each type of cell line, showing the highest levels of expression (as judged by immunofluorescence), was chosen for further analysis.

Detection of the P, V, and NP proteins. Western blot analysis of total extracts of cell lines expressing the P, V, NP, and P plus NP proteins (hereafter referred to as P, V, NP, and P + NP cell lines) (24 h after the removal of a tetracycline block) demonstrated that the cells made full-length authentic proteins (Fig. 3; it should be noted that MAb SV5-NPd, which was used to detect the NP protein, reacts only weakly in Western blots, and thus the comparative intensities of the NP and P bands are not a quantitative reflection of the ratio of the two proteins). The expression of the genes was clearly under the control of tTA. This control was very tight in the case of the NP and P + NP cell lines, as these proteins could not be detected in cells which had been grown in the presence of tetracycline. In the P and V cell lines, there was a basal level of P and V synthesis in the presence of tetracycline. However, upon induction, the level of expression these proteins increased significantly. The low level of production of the P and V proteins in the presence of tetracycline presumably reflects the insertion of the P and V genes near weak cellular promoters.

To further investigate the tetracycline control of P, V, and NP gene expression, cells that had been grown in the presence or absence (for 24 h) of tetracycline were labelled for 2 h with L-[^{35}S]methionine, and the P, V, and NP proteins were immunoprecipitated with MAbs specific for these proteins. In addition, cells were released from the tetracycline block for 24 h, reincubated in the presence of tetracycline for 24 h, and then labelled with [^{35}S]methionine, and the viral proteins were immunoprecipitated. These results confirmed the Western blot results in that no viral proteins could be detected in the NP and P + NP cells that had been grown continuously in the presence of tetracycline. However, low levels of the P and V proteins could be detected in cells that had been grown continuously in the presence of tetracycline. Following release from tetracycline for 24 h, inducible expression of the tTA-responsive genes in all the cell lines occurred. Furthermore, following reincubation of the cells with tetracycline, gene expression was down-regulated (Fig. 4). It should also be noted that while the levels of P and V protein synthesis returned to basal levels, a low level of NP expression could still be detected 24 h postinhibition, possibly reflecting differences in the stability of the P, V, and NP mRNAs.

Kinetics of gene induction following release from tetracycline. A time course of expression, following release from tetracycline, was carried out on the selected cell lines by monitoring the proportion of cells that became positive, with time, for expression (as judged by cells positive by immunofluorescence for the P, V, and/or NP proteins as a proportion of cells present in a field; e.g., Fig. 5). There were significant differences in the kinetics of induction of the tTA-responsive genes in the different cell lines (Table 2). In addition, there was asynchrony in the expression of these genes within a given cell population. For example, there was a relatively rapid induction of P expression in the P cell line, with more than 80% of the cells showing fluorescence by 12 h postrelease. (It should also be noted that low-level P expression could be detected in a small proportion of the cells in the presence of tetracycline). The kinetics of induction of V synthesis was similar. With regard to the NP cell line, there was a gradual increase in the percentage of cells expressing the NP protein, and by 24 h postrelease, 60 to 80% of the cells were clearly positive by fluorescence. However, the induction of both the NP and P genes in the P + NP cell line was much slower, and by 24 h postrelease, only 5 to 10% of the cells were positive; this level had reached 60 to 80% positive by 72 h postrelease. It should also be noted that >90% of the cells that were positive for NP were also positive for P and vice versa (see also Fig. 8).

Although the level of induction of the tTA-responsive genes could be >90%, there were always some cells that appeared negative by immunofluorescence, regardless of the length of time of release from the tetracycline block. There was also some variation from experiment to experiment in the percentage of cells which became positive following release (data not shown). Furthermore, in cells that had been passaged for more

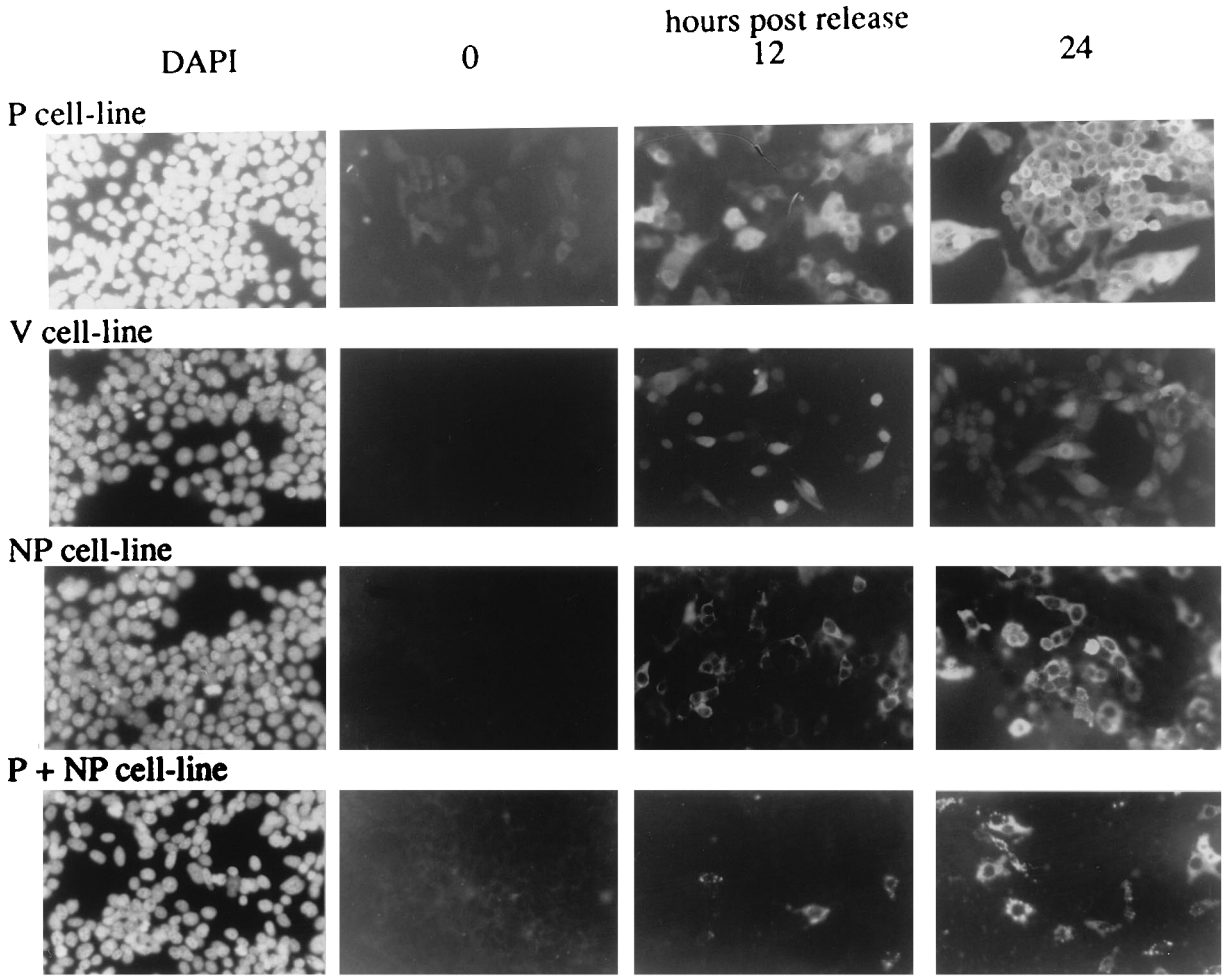

FIG. 5. Photographs showing the presence of the P, V, and NP proteins in individual BF cells expressing the P, V, NP, and P plus NP proteins with time (0, 12, and 24 h) following release from the tetracycline block. Also shown is the DAPI staining of a typical field. Monolayers were fixed and stained with an MAb specific for the P, V, or NP protein by using direct immunofluorescence. FITC-labelled MAb SV5-Pk was used to detect the P and V proteins, while rhodamine-labelled SV5-NPa was used to detect NP. Only the pattern obtained with SV5-NPa is shown for the P + NP cell line, but cells that were positive for NP were nearly always positive for P and vice versa (data not shown; see also Fig. 8).

than 3 weeks in the absence of tetracycline (the expression of the NP and P proteins was not highly toxic to the cells), there were still between 5 and 20% of cells that were negative for expression. This was not because the negative cells had lost either the ability to express tTA or the tTA-responsive genes, since following cloning, all subclones retained the same properties of the original cell lines (data not shown).

TABLE 2. Percentage of cells accumulating with time, following release from a tetracycline block, that were positive by immunofluorescence for the P, V, or NP protein

Time (h) postrelease	% Positive cells			
	NP	P	V	P + NP
0	0	—[a]	—	0
3	0	—	—	0
6	0.5–1	40–60	30–50	<0.1
12	5–10	50–80	50–70	1–5
18	20–30	>90	>90	5–10
24	60–80	>90	>90	5–10
48	70–90	>90	ND	25–30
72	ND[b]	ND	ND	60–80

[a] —, some weak fluorescence observed.
[b] ND, not done.

Asynchrony of expression. In the P + NP cell line, although there was marked asynchrony in the time in which individual cells expressed the viral proteins following release from the tetracycline block, the vast majority of cells were either positive or negative for both viral proteins, and cells expressing one but not the other were rare (see Fig. 8). The NP and P genes were originally on different plasmids, which would suggest that both genes were available for activation by tTA at the same time and that variations in the level of the tTA protein were responsible for the asynchrony observed.

To address further the reasons for asynchronous expression of responder genes, cells were stained for the presence of the tTA protein by immunofluorescence. These results clearly demonstrated that there were marked differences in the levels of the tTA protein between the cell lines and between individual cells in a given cell line. The tTA protein could most easily be detected in the P and V cell lines but, even here, there was variation in the level of expression that could be detected in individual cells (Fig. 6). In general, cells that had high levels of

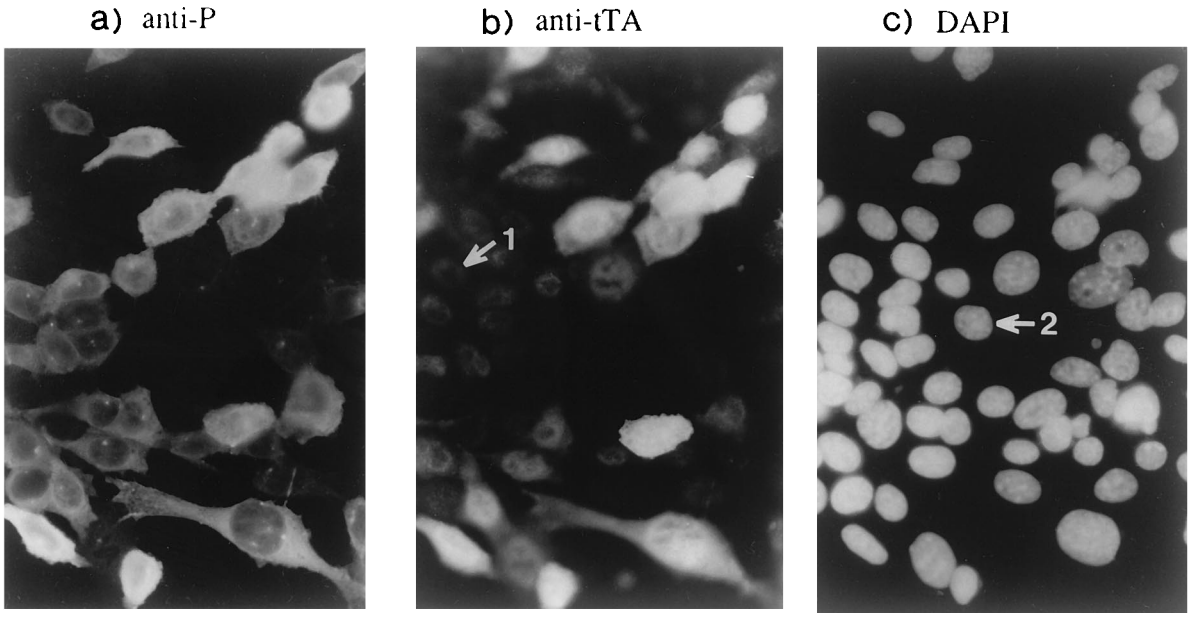

FIG. 6. Photographs showing the intracellular distribution of the tTA protein in the P cell line that had been released from a tetracycline block for 24 h. The cells were also stained for P and with DAPI. A cell weakly positive for tTA but negative for P is indicated by arrow 1, and a cell negative for both tTA and P is indicated by arrow 2. Monolayers were fixed and stained with rhodamine by indirect fluorescence with the rabbit anti-tTA antiserum and then by direct fluorescence with an FITC-labelled MAb specific for P. The MAb used to detect P was SV5-Pk, an antibody that also binds to V and shows no background binding against naive cells (as can be seen from the completely negative cells in Fig. 6; see also Fig. 5 and 9).

tTA were positive for the induced proteins (following release from tetracycline), while cells that had no detectable tTA were usually negative for the induced proteins. However, these correlations were not absolute, and cells which appeared positive for tTA sometimes appeared negative for the inducible proteins.

Phosphorylation of the P and V proteins. The P and V proteins of paramyxoviruses are known to be highly phosphorylated. In SV5-infected cells, both proteins can be visualized by labelling the cells with [^{32}P]phosphate and then subjecting them to immunoprecipitation (data not shown). It has been suggested that the L protein may phosphorylate the P protein of Sendai virus (8), although when the P and V proteins of Sendai virus were expressed in transfected cells, they appeared to be highly phosphorylated (7). It was thus of interest to determine whether the P and V proteins of SV5 were also phosphorylated in the absence of L. It can be clearly seen in Fig. 7 that both P and V are phosphorylated in the respective

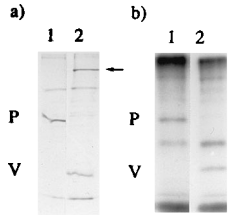

FIG. 7. Analysis of radioactively labelled polypeptides present in immunoprecipitates formed by the reaction of MAb SV5-Pk with soluble antigen extracts made from BF cell lines expressing P (lanes 1) and V (lanes 2). Cells were released from the tetracycline block for 24 h before being radioactively labelled with either [^{35}S]methionine (a) or [^{32}P]phosphate (b) for 2 h prior to extraction. Polypeptides were separated by electrophoresis through an SDS–12% polyacrylamide slab gel and visualized by autoradiography. Note that MAb SV5-Pk reacts with an epitope on the common N-terminal domains of P and V, and thus the 150K host cell protein is coprecipitated only with V.

cell lines. It should also be noted from Fig. 7 that a cellular protein with an estimated molecular weight of 150,000 (150K protein; marked with an arrow in Fig. 7a, lane 2) is coprecipitated with the V protein but not the P protein (MAb SV5-Pk was used to precipitate both the P and V proteins). It is also clear from Fig. 7 that the 150K host protein is not phosphorylated. The 150K protein can also be visualized coprecipitating with the V protein in Fig. 4 and is not precipitated with other MAbs (Fig. 4 and data not shown).

Intracellular localization of the P, V, and NP proteins. Examination of the cell lines by immunofluorescence and confocal microscopy revealed distinct differences in the distributions of the P, V, and NP proteins when expressed separately. When expressed alone, the P protein generally had a more diffuse cytoplasmic distribution, although in some cells P could also be visualized in an extremely fine pattern of filament-like structures. Furthermore, in the majority of cells, P could also be seen accumulating at a single focal point (occasionally two) close to or on the nuclear membrane (Fig. 8a and 6a). In contrast, the related V protein had primarily a diffuse nuclear distribution, although when expressed at high levels, it could also be visualized in the cytoplasm (Fig. 8b). The NP protein was distributed throughout the cytoplasm in small cytoplasmic aggregates, giving rise to punctate and granular fluorescence (Fig. 8c). However, when the NP and P proteins were coexpressed, their distribution was clearly very different. The most striking difference was that they colocalized in large cytoplasmic inclusion bodies (Fig. 8c and d) that were similar in appearance to those visualized in virus-infected cells (12). However, in a proportion of cells they could also be visualized more evenly distributed throughout the cytoplasm, but even here they clearly colocalized, often in smaller aggregates. It was not possible to monitor the kinetics of formation of the inclusion bodies with time, as there was asynchronous expression between individual cells (as detailed in Table 2, although it should also be noted that cells were either positive or negative

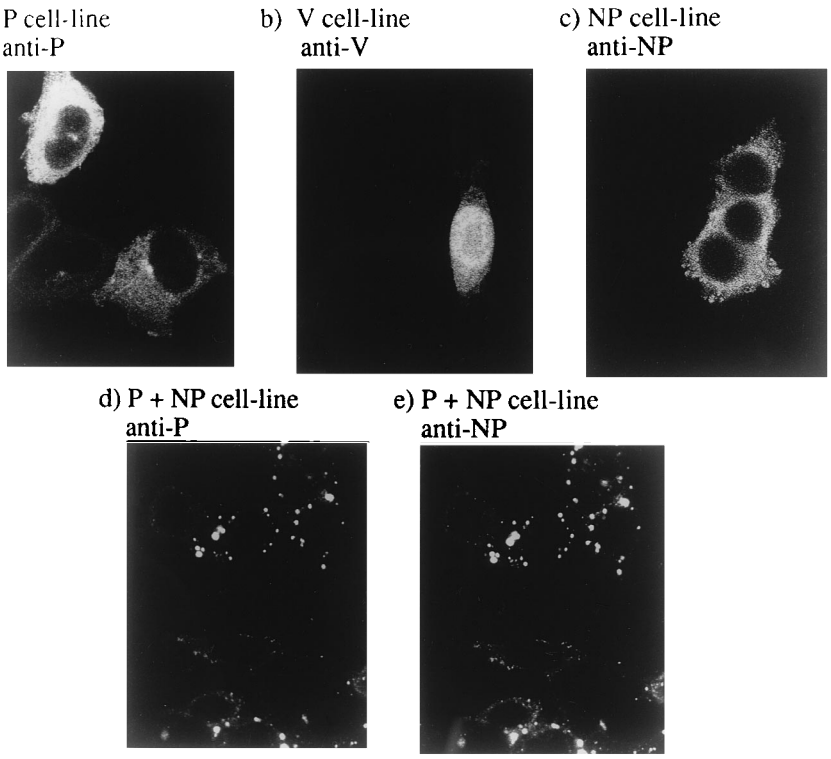

FIG. 8. Photographs illustrating the intracellular distribution of P, V, and NP in cells that had been released from a tetracycline block for 24 h and were expressing the P, V, NP, or P plus NP proteins, as visualized by confocal microscopy. Monolayers were fixed and stained by direct fluorescence using FITC-labelled SV5-Pk, which detects both P and V, and rhodamine-labelled SV5-NPa, which detects NP. Cells expressing P plus NP were stained simultaneously with both antibodies.

for both P and NP). Nevertheless, it is clear that the appearance of these inclusion bodies must occur relatively soon after coexpression of the two proteins, since at early times following release from the tetracycline block, inclusion bodies could be visualized in the small number of cells positive for the P and NP proteins (data not shown).

To determine whether the V and NP proteins colocalized when expressed together, a permanent cell line that expressed the NP and V proteins was isolated. This was achieved by transfecting the V cell line with plasmid pUHD 10-3SV5/NP in the presence of a second plasmid which conferred resistance to hygromycin. Colonies of cells that grew in the presence of hygromycin were selected and examined for expression of the NP and V proteins by immunofluorescence. Examination of these cells clearly revealed that coexpression of NP and V resulted in a partial redistribution of the NP protein compared with when it was expressed alone. Thus, 24 h after release from the tetracycline block, when coexpressed, both NP and V had a cytoplasmic and nuclear distribution but there was no colocalization of NP and V into inclusion bodies (as occurred when P and NP were coexpressed). Furthermore, although in some cells NP could be seen to be self-aggregating, in general it had a diffuse nonaggregated distribution when colocalized with V (Fig. 9). However, with increasing time postrelease from the tetracycline block, more NP could be detected in small cytoplasmic aggregates. This may be due to the much longer half-life of NP than of V (34).

NP-P and NP-V protein-protein interactions. To further examine the interaction of NP with P and V, we developed a method for directly visualizing protein-protein complex formation. Monolayers of BF cells expressing either the P or V protein were fixed and permeabilized as for immunofluorescence. These cells were then incubated with soluble antigen extracts of NP cells that had been disrupted in PBS. Following binding, the presence of the NP and P or V proteins was visualized by immunofluorescence (Fig. 10). These results clearly demonstrated that the NP protein bound to BF cells expressing either the P or V protein but not to control BF cells. It should also be noted from Fig. 10 that following binding of NP to the P cells, the NP protein had primarily a cytoplasmic distribution, but after binding to the V cells, it had primarily a nuclear distribution. Furthermore, cells negative for P or V, in the respective cell lines, did not bind NP. The presence of 0.1% Nonidet P-40 prevented the binding of NP to V cells. In contrast, NP bound to P in the presence of 0.5% Nonidet P-40, and even, albeit less strongly, in the presence of 0.5% Nonidet P-40 plus 0.1% SDS (data not shown). In the absence of detergent, NP bound to P and V in the presence of 2 M NaCl, and binding could be visualized even in the presence of 4 M NaCl, but again at reduced levels.

We also performed competitive binding experiments in which soluble antigen extracts of cells containing NP were mixed (for 2 h at 4°C) with extracts of cells containing the P or V protein prior to incubation of the mixture with fixed and permeabilized cells expressing either the P or V protein. These results clearly demonstrated that preincubation of P with NP blocked the binding of NP to both P and V cells, but that preincubation of V with NP, while blocking the binding of NP to V cells, did not block the binding to P cells (data not shown).

DISCUSSION

A major consideration in constructing plasmid pMR101/tTA, which encodes the tTA-Neo fusion protein, was the hope

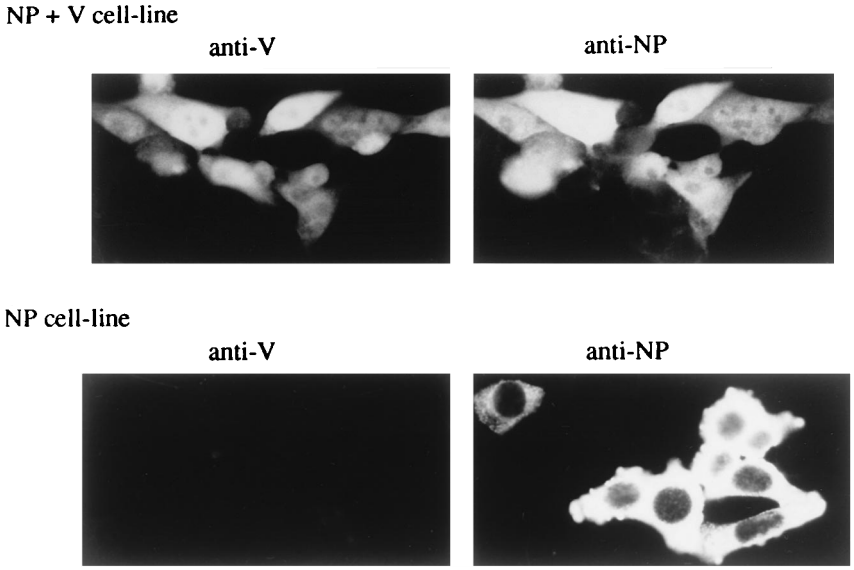

FIG. 9. Photographs comparing the distributions of the NP protein in NP and NP + V cell lines. Monolayers were release from the tetracycline block for 24 h, fixed, and stained by direct fluorescence with FITC-labelled SV5-Pk, to detect V, and rhodamine-labelled SV5-NPa, to detect NP.

that after selection of transfected cells with Geneticin, resistant cells would continuously make the tTA-Neo fusion protein at sufficiently high levels, regardless of their physiological state or their position in the cell cycle, to be able to activate at all times tTA-responsive promoters. However, not only did the different cell lines isolated make different amounts of the tTA protein, but immunofluorescence analysis indicated that the level of the tTA protein in individual cells within a given population also varied. Nevertheless, the advantages of using pMR101/tTA to establish cell lines expressing tTA are that (i) all cells isolated that are resistant to Geneticin also express tTA, albeit to different levels, and (ii) by passaging cells in the presence of Geneticin, the gene will not be inactivated (e.g., by hypermethylation or gene deletion) over a period of time. Indeed, using this approach, we have isolated a variety of cell lines (BF [see above], BHK, Vero, and 293) expressing tTA. The results presented here also demonstrate that high-level expression of paramyxovirus proteins can be achieved in mammalian cells by using the tTA system, and we suggest that such systems may prove extremely useful, noncytocidal alternatives to the vaccinia virus T7 polymerase system for making viral proteins required to carry out reverse genetics studies on these viruses.

Although the intracellular distribution of virus proteins will be effected by the actual process of virus replication, studies examining their intracellular localization when expressed alone or in combination have proved revealing. When expressed alone, the NP protein was present in small cytoplasmic aggregates, formed presumably through its propensity to self-aggregate (2, 12, 32). In contrast, the P protein had a more diffuse cytoplasmic distribution. Furthermore, P could also be visualized in some cells as an extremely fine pattern of filament-like structures and also often appeared to specifically locate in structures close to the nuclear membrane that, in appearance, size, and distribution, were similar to centromeres (Fig. 6). Intriguingly, when expressed alone, V had primarily a nuclear localization, although it could also be seen in the cytoplasm of cells expressing high levels of the protein. Paterson et al. (26), using MAbs specific for V, reported that while V could be detected throughout the cytoplasm of SV5-infected cells, giving rise to diffuse cytoplasmic immunofluorescence, it could also be detected in nuclei of infected cells, giving diffuse nuclear staining. Thus, V may be a multifunctional protein with different biological roles. Whether there is any functional consequences for the nuclear localization of V is currently under investigation, as is its affinity for the 150K host cell protein of unknown function.

It was evident from comparing the cellular distributions of the NP, P, and V proteins when expressed alone or together that NP associated with both P and V. Furthermore, the method presented here for directly visualizing protein-protein interactions also clearly demonstrated that NP bound to cells expressing either the P or V protein but not to naive cells (Fig. 10). The bindings of NP to P and V were clearly different in that NP-V binding was inhibited by the presence of nonionic detergents but NP-P interactions were resistant. Evidence has been presented that there are two NP binding sites on the P protein of rabies virus (3). It has also been suggested that the P protein of Sendai virus may contain two NP binding sites: a strong binding site at its C terminus and a weak binding site at its N terminus (which would be common to V and W) (5). However, no direct evidence for the interaction of NP with V or W has yet been reported. The observations presented here are consistent with the idea that there are two NP binding sites on P. Thus, the finding that P could inhibit the binding of NP to V suggests that P and V may have a common binding site for NP, probably at their common N-terminal domain. However, the finding that V did not prevent NP from binding to P suggests either that there must be a second binding site on P for NP or that P has a much higher affinity than V for their common binding site.

It has been suggested that one role of the P protein in paramyxovirus and rhabdovirus infections is to act as a chaperone for NP, keeping it soluble (2, 19) and preventing it from self-aggregating and assembling illegitimately in the absence of virus replication (5). However, as discussed above, the P protein may have two binding sites for NP. Furthermore, it has been suggested that P may exist as trimers in infected cells (24). As the NP protein has a propensity to self-assemble (2, 12, 32), coexpression of the NP and P proteins could lead to the formation of large aggregates, with the P protein cross-

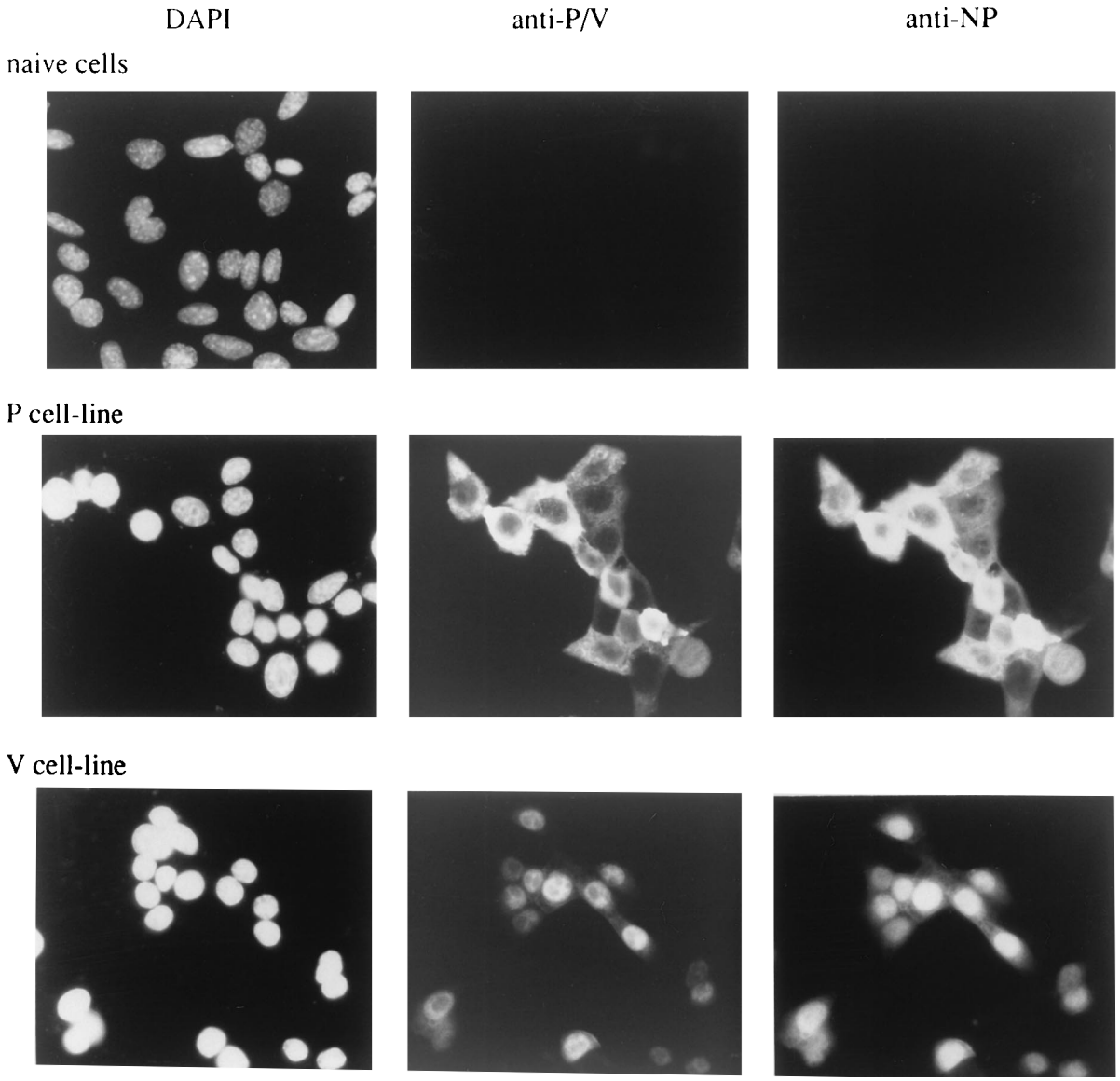

FIG. 10. Photographs illustrating the binding of NP to cells expressing the P or V protein but not to naive cells. A soluble antigen extract was made from cells expressing the NP protein. This extract was incubated with fixed and permeabilized naive cells or with cells that had been released from a tetracycline block for 24 and that expressed either the P or V protein. Cells were stained simultaneously with DAPI together with FITC-labelled SV5-Pk, which detects both P and V, and rhodamine-labelled SV5-NPa, which detects NP. Note that at 24 h postinduction, not all cells released from the tetracycline block were expressing the P or V protein and that the NP protein did not bind to these negative cells.

linking any self-assembled NP complexes. Indeed the experiments presented here clearly demonstrate that when P and NP were coexpressed (in the absence of ongoing virus replication), they aggregated into large cytoplasmic inclusions bodies, similar to those visualized in SV5-infected cells (11). Coexpression of the NP and P proteins in the rabies and respiratory syncytial virus systems also resulted in the formation of cytoplasmic inclusion bodies similar to those reported here (3, 14). Therefore, at least with these viruses, it is likely that other virus proteins may also be involved in keeping the NP protein soluble prior to its assembly into nucleocapsids. The results presented here suggest a role for V, or other N-terminal P-related proteins with only one binding site for NP, in this process. Thus, NP and V clearly associate but do not form inclusion bodies, and V can influences the distribution of NP within a cell. Since P is part of the polymerase complex and forms stable protein-protein interactions with L (28), it may be, at least with SV5-like viruses, that V delivers soluble NP to the P protein in the polymerase complex as part of the process for encapsidating newly synthesized viral RNA. It is also of note that with SV5, in which the V gene is in genomic sense, during transcription significantly more V mRNA than P mRNA is synthesized (26b). Such a mechanism ensures that, at least at early times postinfection, there is more V than P, a prerequisite if one of the roles of V is keep NP soluble by preventing it from either self-associating or forming large heteroaggregates with P in the absence of viral RNA synthesis.

There are obviously some discrepancies between the conclusions drawn here and those of Curran et al. (5), which suggest that Sendai virus P acts as a chaperone keeping NP soluble. While these difference will be resolved by further experimentation, it is possible that P and V do not play exactly the same

roles in virus transcription and replication in Sendai-like viruses and SV5-like viruses.

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