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Individual Expression of Influenza Virus PA Protein Induces Degradation of Coexpressed Proteins

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In the process of in vivo reconstitution of influenza virus transcriptase-replicase complex, an inhibitory effect was observed when the level of PA protein expression was increased. This inhibition was paralleled by a decrease in the accumulation of the other influenza virus core proteins. The sole expression of PA protein was sufficient to reduce the accumulation level of the proteins encoded by the coexpressed genes. The PA effect was observed upon influenza virus and non-influenza virus proteins and independently of the expression system chosen and the origin of cell line used. The expression of PA protein did not induce variations in the translation of the target proteins but did induce variations on their half-lives, which were clearly reduced. A functional PA subunit seems to be necessary to induce this negative effect, because an inactive point mutant was unable to decrease the steady-state levels or the half-lives of the reporter proteins. The PA effect was observed as early as 5 h after its expression, and continuous synthesis of proteins was not required for performance of its biological activity. The results presented represent the first biological activity of individually expressed PA polymerase subunit.

The genome of influenza type A viruses consists of a set of eight single-stranded RNA segments of negative polarity, encoding a total of 10 genes (for a review, see reference 20). For the genome to be expressed, it has to form ribonucleoprotein (RNP) complexes in which four viral proteins are essential constituents: the nucleoprotein (NP) and the three subunits of the polymerase (PB1, PB2, and PA) (reviewed in reference 18). Upon entry into a susceptible cell, these RNPs are transported to the nucleus, where transcription and replication take place (13). Transcription initiation makes use of capped oligonucleotides generated by endonucleolytic cleavage of cellular pre-mRNAs (19). These primers are elongated until the polymerase reaches a five- to seven-U stretch close to the 5′ end of the negative-strand template, which serves as a polyadenylation signal (23, 30). Thus, viral mRNAs that contain additional sequences at the 5′ end and lack certain viral sequences at the 3′ end are produced. Replication of viral RNAs involves the synthesis of positive-strand replicative intermediates that are exact copies of the virion RNAs (12) and form RNP complexes similar to the virion RNPs. RNA replication also takes place in the nucleus, in close association with the nuclear matrix (16, 21), and requires the presence of newly synthesized NP (31).

The roles of the different polymerase subunits in these processes have been partly outlined. The PB1 subunit contains several sequence motifs typical of the viral RNA-dependent RNA polymerases sequences (9, 29), which have been shown essential for viral RNA synthesis (4). In agreement with these observations, the PB1 protein can be cross-linked to the ribonucleoside triphosphate precursor and has been proposed as responsible for the polymerizing activity in the complex (6). Likewise, the PB2 subunit can be cross-linked to cap analogs (5, 33) and may be involved in transcription initiation. On the contrary, no clear function for the PA subunit has been proposed. The phenotype of temperature-sensitive mutants in the PA-encoding gene suggests a role in virion RNA synthesis (reviewed in reference 24), although no biochemical activity has been associated with the PA protein, in spite of its absolute requirement for viral RNA expression in systems reconstituted in vivo (8, 14, 26). Two lines of evidence suggest that PA protein may be toxic when it is singly expressed in mammalian cells. First, trials to produce stably expression of the PA gene in A2G mouse kidney tumor cells have been unsuccessful, since transfected cells expressing PA stop growing after a few days (32). Second, using SVPA recombinants, we have observed that nuclear location of PA protein correlated with chromatin condensation and aberrant nuclear morphology (28).

Taking into account these observations, we decided to study the individual role of the PA subunit, as well as to characterize the potential negative effect of PA and the mechanism involved in this biological activity. We found that the expression of PA protein from cloned DNA leads to a generalized reduction in the accumulation of coexpressed proteins. This effect is observed both for viral and nonviral proteins and is mediated by a reduction in their half-lives.

MATERIALS AND METHODS

Cells, viruses, and plasmids. The COS-1 cell line (11) was obtained from Y. Gluzman. The HeLa and the N2A cell lines were purchased from the American Type Culture Collection. Cell cultures were grown in Dulbecco's modified Eagle medium (DMEM) containing 5% (COS-1 and HeLa) or 10% (N2A) fetal bovine serum. For N2A cells, the medium was supplemented with 2 mM glutamine.

Vaccinia virus VTF7-3 is a recombinant virus that expresses the phage T7 RNA polymerase (10). The simian virus 40 (SV40) recombinant viruses SVPB1, SVPB2, SVPA, and SVNP, which express the influenza virus polymerase subunits and nucleoprotein of strain A/Victoria/75, have been described elsewhere (7, 28). The SVPA154 recombinant virus expresses a point-mutated form of PA protein (a Glu-to-Gly change at position 154). This mutant protein is not able to move to the nucleus (27), and it does not support virus-like RNA expression in a reconstituted system (6a).

All plasmids used in this study contained a T7 RNA polymerase promoter upstream of the cloned gene to drive transcription by this enzyme. pGEM3 recombinant plasmids encoding the influenza virus polymerase and NP proteins (pGPB1, pGPB2, pGPA, and pGNP) have been described elsewhere (26). A similar plasmid encoding influenza virus NS1 protein (pGNS1 [25a]) was also used. Plasmid pARPA154 encodes the point-mutated inactive form of PA protein (7). Plasmid pBSHT-9.1 encodes the α′ subunit of human casein kinase II

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(CKIIα′) (22). Plasmid pT7 EMC-CAT, a generous gift of T. Zürcher, contains the chloramphenicol acetyltransferase (*cat*) gene downstream of the internal ribosome entry sequence of encephalomyocarditis virus. Plasmid pPB2CAT-9, a gift of M. Krystal, contains the *cat* gene in negative polarity flanked by the 5′- and 3′-terminal noncoding sequences of the influenza virus RNA segment 1.

Transfection. Subconfluent monolayers of cells were infected with VTF7-3 virus at a multiplicity of infection of 5 to 10 PFU per cell. After 1 h of adsorption at 37°C, cells were transfected with the indicated mixture of plasmids by the liposome-mediated method, using the Lipofectin reagent (Gibco BRL) according to the manufacturer's recommendations (2 μl of Lipofectin per μg of DNA). The total amount of transfected DNA per dish was kept constant, adjusting if necessary with pGEM3 plasmid to avoid differences in transfection efficiency. Cells were incubated at 37°C in serum-free DMEM until the corresponding harvesting time (usually 16 h posttransfection). Different plasmid DNA preparations were used, and experiments were repeated at least three times; the figures show results representative experiments.

For the in vivo reconstitution of active synthetic influenza virus-like RNPs, the *cat* gene was used as a reporter (26). Cells infected with VTF7-3 virus and transfected with plasmids pGPB1, pGPB2, pGPA, and pGNP were transfected 6 h later with an in vitro-transcribed influenza virus-like CAT RNA (1 μg) derived from plasmid pPB2CAT9. After 16 h of incubation, cells were harvested and the cell extracts were tested for CAT activity. The influenza virus-like CAT RNA template was prepared by using the Ambion Megascript transcription kit by incubating 1 μg of *Hga*I-digested pPB2CAT-9 with 15 U of T7 RNA polymerase in the manufacturer's buffer. Transcription mixtures were then incubated for 15 min with DNase I and directly used for transfection into infected-transfected cell cultures.

CAT assays. For CAT assays, total cell extracts were prepared in 0.25 M Tris HCl (pH 7.5) by three cycles of freezing-thawing. Protein concentration was determined by the bicinchoninic acid method (Pierce). CAT activity was analyzed by the phase extraction method, measuring the transfer of ^{3}H-acetyl moieties from ^{3}H-acetyl coenzyme (acetyl-CoA) to unlabelled chloramphenicol and separating the labelled products by extraction of acetylchloramphenicol into the organic phase. The reactions were carried out in a final volume of 50 μl containing 5 to 10 μl of cell extract, 0.062 μCi of ^{3}H-acetyl-CoA (Amersham), 200 μM chloramphenicol, 200 μM acetyl-CoA, and 50 mM Tris HCl (pH 7.5). Incubation was for 1 h at 37°C and was stopped by adding 250 μl of a 100 mM sodium borate–5 M NaCl (pH 9) solution. Upon addition of 1.5 ml of liquid scintillation cocktail (Optifluor O; Packard), radioactivity in the organic phase was determined in an LKB liquid scintillation counter.

Western blotting (immunoblotting). For Western blot assays, total cell extracts were used. Cells were resuspended directly in sample loading buffer, and the proteins were resolved by electrophoresis in sodium dodecyl sulfate (SDS)-polyacrylamide gels (25). Gels were then transferred to Immobilon membranes (Millipore) in 3 mM Na_2CO_3–10 mM $NaHCO_3$–20% (vol/vol) methanol buffer at 150 mA for 1 h. After blocking with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h at room temperature, the filters were incubated for 1 h at room temperature with the corresponding primary antibodies diluted in PBS containing 0.25% Tween 20 and 0.1% BSA. The filters were washed for 15 min in PBS plus 0.25% Tween 20 and incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies (goat anti-mouse–peroxidase or protein A-peroxidase, 1/3,000 dilution; BioRad). Finally, the filters were washed in PBS plus 0.25% Tween 20 for 30 min, and the specific bands were visualized with enhanced chemiluminescence (ECL system; Amersham). The following antibodies were used: for PB2 protein, PARB2 8N, a rabbit antiserum that was prepared by immunizing animals with a carboxyl-truncated form of PB2 (1/100 dilution); for PA protein, monoclonal antibody (MAb) 12, a MAb made against *Escherichia coli*-expressed PA (1/3 dilution from culture supernatant) (2); for NP protein, a rabbit antiserum prepared against purified viral RNPs (1/200 dilution); for PB1 protein, antipeptide serum 574, a rabbit antiserum prepared by intradermal immunization of rabbits with purified MPB1/1 peptide (positions 70 to 81 in the PB1 sequence) (1/10 dilution); for NS1 protein, rabbit anti-NS1 serum prepared by hyperimmunzation of rabbits with purified protein (1/300 dilution); and for CKIIα′, an anti-human casein kinase II (CKII) antipeptide (positions 70 to 91 of the α subunit sequence; Upstate Biotechnology, Inc.) (working concentration, 1 μg/ml).

Pulse-chase experiments. Cultures of COS-1 cells, infected and transfected in 16-mm-diameter dishes as described above, were starved for 90 min in methionine and cysteine-free DMEM at 16 h posttransfection and labelled for 15 min with a mixture of [^{35}S]Met and [^{35}S]Cys (Promix; Amersham) in 100 μl of the same medium. The chase was carried out by extensive washing with DMEM and incubation for the indicated times in 500 μl of DMEM supplemented with 10% fetal bovine serum, 2 mM methionine, 2 mM cysteine, and, when indicated, 100 μg of cycloheximide (Sigma) per ml. Finally, cells were washed with PBS and lysed in 500 μl of radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% Nonidet P-40 [NP-40], 0.5% deoxycholate, 0.1% SDS, 50 mM Tris HCl [pH 7.5]).

Immunoprecipitation. For immunoprecipitation of PB2 protein, protein A-Sepharose was incubated for 2 h at room temperature in 100 mM NaCl–5 mM EDTA–50 mM Tris (pH 7.5) (TNE)–1% NP-40 buffer with rabbit anti-mouse serum (Nordic Immunology) at 1/60 dilution. The protein A-Sepharose–rabbit anti-mouse complexes were washed three times with TNE–1% NP-40 and incubated in the same buffer for 2 h at room temperature with MAb 20, a specific MAb against PB2 protein (2), in a 1/150 dilution from an ascites fluid. The immunoconjugates were washed three times in TNE–1% NP-40 and once in RIPA buffer and were incubated overnight at 4°C with 250 μl of total cell extracts in RIPA buffer. The immunoprecipitates were then washed three times with RIPA buffer, extracted with sample loading buffer, boiled for 5 min, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) in 10% acrylamide gels. Gels were vacuum dried and exposed for autoradiography.

Immunofluorescence. Subconfluent monolayers of COS-1 cells were infected with the SV40 recombinant viruses expressing influenza virus proteins at multiplicities of infection of 1 to 5 PFU per cell. At 48 h postinfection, the cells were fixed with methanol at −20°C and stored in PBS at 4°C. Cells were saturated with 2% BSA in PBS and incubated for 1 h at room temperature with the following dilutions of specific antibodies in PBS–0.1% BSA: PA-specific MAb 12 (culture supernatant), PB2-specific MAb 25 (1/3 dilution from culture supernatant), and rabbit anti-RNP (1/500 dilution). After being washed with PBS, cells were stained for 1 h at room temperature with a 1/500 dilution of fluorescein-labelled donkey anti-rabbit immunoglobulin antibodies and/or a 1/200 dilution of Texas red-labelled sheep anti-mouse immunoglobulin antibodies (Amersham) in PBS–0.1% BSA. Finally, the preparations were washed with PBS, mounted in Mowiol (Aldrich), and photographed in a Zeiss fluorescence microscope.

RESULTS

In the course of our studies on the biological role of influenza virus PA polymerase subunit, its gene was cloned as SV40 or pGEM3 recombinants (9, 26). We observed that in double-infection experiments using SVPA and SVPB2 recombinant viruses, PA induced a decrease in the expression of PB2 protein, as shown by double-immunofluorescence assays (26a). This result, together with the inability to establish stable cell lines individually expressing PA protein (32) and the alterations in cell morphology after its expression (27), suggested a toxic effect of PA gene expression in cultured cells.

Expression of PA protein diminishes the accumulation of coexpressed influenza virus core proteins and reduces the activity of reconstituted RNA polymerase. For the study of influenza virus RNA transcription and replication, we made use of an in vivo-reconstituted system in which viral RNA polymerase activity is provided by transfection of a set of pGEM3 recombinant plasmids encoding the influenza virus NP and P proteins (plasmids pGNP, pGPB1, pGPB2, and pGPA) into COS-1 cells previously infected with a recombinant vaccinia virus (VTF7-3) expressing the T7 RNA polymerase (26). The functionality of the reconstituted polymerase was determined by measuring CAT activity in cells transfected with an in vitro-synthesized virus-like CAT RNA. This virus-like CAT RNA contains the antisense coding region of the CAT gene flanked by the 5′ and 3′ noncoding sequences of the influenza virus PB2 gene and can therefore be transcribed by the influenza virus polymerase to yield CAT activity.

To optimize the reconstitution system, we performed a series of dose-effect experiments for each of the core proteins, increasing the amount of one of the transfected DNAs while keeping the others at constant levels. We observed that increasing amounts of transfected DNAs coding for PB1, PB2, or NP protein, ranging from 0.5 to 8 μg, clearly increased the amount of CAT activity. In contrast, a strong reduction in CAT activity was observed as the concentration of transfected PA plasmid was increased (Fig. 1A). We next analyzed the actual amounts of influenza virus core proteins that accumulated at the different concentrations of transfected PA-expressing plasmid tested. As shown in Fig. 1C for PB2 and NP, the amounts of these core proteins dropped as the concentration of the transfected pGPA recombinant was increased; the same results were obtained for PB1 protein (data not shown). However, the amounts of the other core proteins remained constant when dose effects for PB1-, PB2-, and NP-encoding DNAs were analyzed (data not shown). These results could be interpreted as the consequence of the action of any possible combination

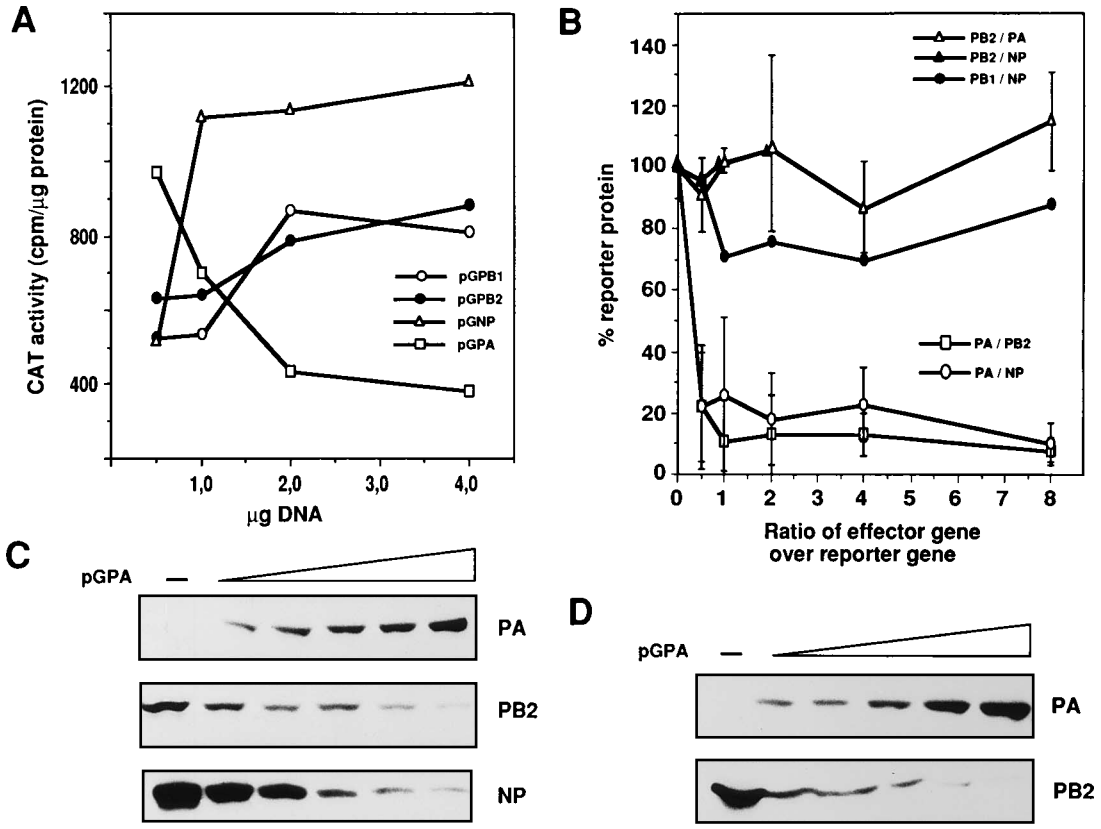

FIG. 1. Increasing amounts of PA protein produce a decrease on influenza virus core protein levels and on virus transcriptase-dependent expression. (A) CAT activity. Subconfluent monolayers of COS-1 cells (35-mm-diameter dishes) were infected with VTF7-3 (multiplicity of infection of 10 PFU per cell) and transfected with the four recombinant pGEM plasmids encoding influenza virus core proteins. Each curve represents a dose-response experiment in which the indicated plasmid was transfected at increasing amounts (0.5, 1, 2, and 4 μg), while the three others were maintained at a constant level (1 μg each). Upon transfection of influenza virus-like CAT RNA, CAT activity was measured as described in Materials and Methods. (B) Quantification of accumulated influenza virus core proteins on cells expressing PA. COS-1 cells were infected with VTF7-3 and transfected with various combinations of pGEM plasmids encoding influenza virus core proteins. Total cell extracts were made, used for Western blots with specific antibodies, and developed by enhanced chemiluminescence as described in Materials and Methods. The enhanced chemiluminescence signals were quantificated by densitometry. Each graph represents a dose-response experiment for the indicated combination of genes, in which the first is the effector gene and the second is the reporter gene. In each point, the percentage of the remaining reporter protein is expressed as a function of the ratio of effector gene over reporter gene. Each error bar represents the standard deviation from 2 to 11 different experiments, depending on the points. (C) Accumulation of influenza virus core proteins, using increasing amounts of pGPA transfected DNA. Cells infected with VTF7-3 were transfected with 0.5 μg each of plasmids pGPB1, pGPB2, and pGNP and no pGPA (lane −) or increasing amounts (0.25, 0.5, 1, 2, and 4 μg) of pGPA. After transfection of influenza virus-like CAT RNA, total cell extracts were made and assayed for Western blot analysis with specific antibodies against the influenza virus core proteins shown at the right. (D) Accumulation of PB2 protein on cells transfected with increasing amounts of pGPA. Cells were infected with VTF7-3 and transfected with 0.5 μg of pGPB2 and no pGPA (lane −) or increasing amounts of pGPA (as in panel C). Total cell extracts were used for Western blots with antibodies against PA and PB2 proteins as indicated.

of the several influenza virus proteins expressed, including the transfected virion-like RNA, or alternatively as the result of the individual activity of PA protein. To distinguish among these possibilities, constant amounts of plasmid pGPB2, pGPB1, or pGNP were transfected together with increasing amounts of plasmid pGPA, and the accumulations of PA and the other influenza virus proteins were determined by Western blot analysis. The results for the cotransfection of plasmids expressing PA and PB2 are shown in Fig. 1D and indicate that the sole expression of PA protein is sufficient to bring about a dramatic effect on the accumulation of PB2 protein. Similar results were obtained for NP and PB1 proteins (data not shown).

The quantitative information of the PA protein effect on coexpressed protein accumulation is shown in Fig. 1B. The results of several cotransfection experiments, in which different combinations of effector and reporter genes were used, are presented as the percentage of reporter protein remaining after coexpression of the effector gene compared with that obtained when the reporter gene was expressed individually. As can be observed, when genes other than the PA gene were used as effector genes, the accumulation of the reporter protein ranged from 70 to 140% of the control level. On the contrary, when the PA gene was used as the effector, a dramatic decrease of any reporter protein accumulation was observed, down to values in the range of 5 to 20% of the control level.

The PA effect requires a functional protein and is independent of the expression system used. To eliminate the possibility that the observed effect mediated by PA protein was a nonspecific result of the vaccinia virus expression system used, PA activity was tested in a different system by using SV40 recombinant viruses expressing the PA, PB2, or NP gene (28). To this end, we infected COS-1 cells with SVNP and either the SVPA or SVPB2 recombinant and studied the accumulation of NP by double-immunofluorescence analysis. We preferred to carry out immunofluorescence instead of immunoblotting studies in this expression system in order to avoid the problems derived from the proportion of cells doubly infected with the two SV40 recombinants, which could mask the PA effect on NP accumu-

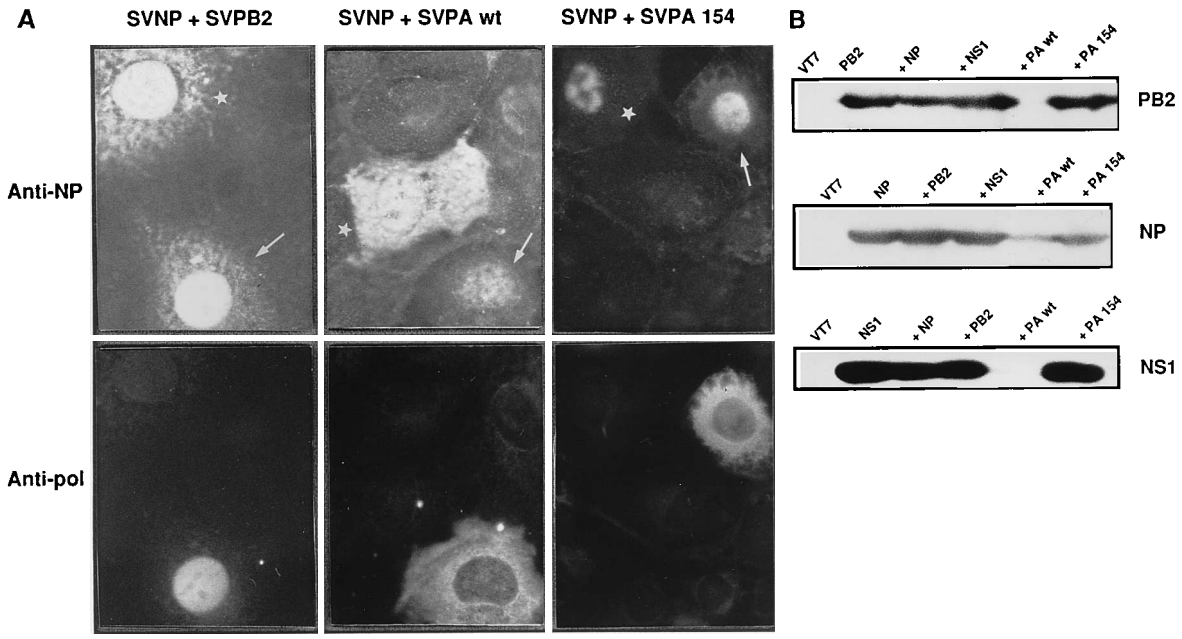

FIG. 2. PA effect is independent of the expression system used and requires a functional protein. (A) SV40 recombinant-mediated expression. COS-1 cells were doubly infected with SVNP and either SVPB2, wild-type SVPA (SVPA wt), or SVPA154 as indicated. After 48 h of incubation, cells were fixed and double-immunofluorescence assays were performed with anti-RNP serum in combination with either an anti-PB2 or anti-PA MAb. Singly infected cells expressing NP alone are marked with stars, while doubly infected cells expressing NP plus PB2, wild-type PA, or PA154 are marked with arrows. For each double infection, both top and bottom rows show the same field. pol, polymerase. (B) T7-mediated expression. COS-1 cells were infected with VTF7-3 and transfected with 0.25 μg of each of the indicated plasmids. When necessary (transfection of reporter plasmids alone), plasmid pGEM-3 was included to keep constant the total amount of transfected DNA. After 16 h of incubation, total cell extracts were analyzed by Western blotting with the corresponding antibodies as indicated at the right.

lation. The results are shown in Fig. 2A. The top row shows the NP immunofluorescence obtained by using a polyclonal antibody, while the bottom row shows the same field stained for PA or PB2 by using specific MAbs. A decrease in NP protein accumulation can be observed when doubly infected cells expressing PA and NP are compared with cells singly infected with SVNP or doubly infected with SVNP and SVPB2 recombinants, used as a control.

We next studied the specificity of the PA effect on protein accumulation. To that end, we used a point mutant of the PA gene which was rendered completely nonfunctional in the CAT assay by a Glu-to-Gly residue substitution at position 154 (data not shown). The accumulation levels of this mutated PA are similar to those found for the wild-type protein (data not shown). Plasmid pARPA154 was transfected into COS-1 cells previously infected with VTF7-3 virus, together with plasmids encoding PB2, NP, or NS1 protein, used as reporters. We included in this case plasmid encoding NS1 to study the possible effect of PA over non-core influenza virus proteins. All possible combinations of the reporter plasmids were doubly transfected and used as controls. Alternatively, COS-1 cells were infected with PA mutant SV40 recombinant plus SVNP recombinant, to examine the level of NP in the doubly infected cells by using immunofluorescence. As shown in Fig. 2, no alteration on the steady-state levels of any of the proteins used as reporters was detected when the PA154 mutant protein was used, and this effect was independent of the expression system used.

These results indicate that a functional PA protein is required for the inhibitory effect to take place and, furthermore, make it very unlikely that this effect was the result of a competition for the genes coexpressed at either the transcriptional or translational level. Moreover, the fact that the action of PA was observed with expression systems as disparate as vaccinia virus T7 and SV40 supports the notion that it is the biological result of the expression of PA protein.

PA protein affects the steady-state levels of non-influenza virus proteins. Our previous studies were carried out with influenza virus proteins as reporters for the PA biological effect. An interaction of PA subunit and the reporter proteins might therefore be required to induce its effect. To deal with this problem, we studied the effect of PA on the accumulation of several proteins not related to influenza virus. To do so, the coding sequences of CAT or CKIIα′ were cloned under the control of a T7 RNA polymerase promoter. The α subunit of CKII instead of the α′ subunit is expressed in COS-1 cells, as detected by immunoblotting with a polyclonal antibody able to recognize both subunits. Subsequently, COS-1 cells were infected with VTF7-3 virus and transfected with either of those plasmids and increasing amounts of plasmid pGPA or pARPA154. The accumulation of CAT enzyme was studied by enzymatic measurements, and that of CKIIα′ was determined by Western blotting. The results are presented in Fig. 3, where a clear reduction on the accumulation of both non-influenza virus proteins can be observed when coexpressed with a wild-type PA but not with a PA154 mutant protein. Quantification studies from three different experiments have been carried out, and we have shown that wild-type PA reduces CKIIα′ levels to 10% of the value obtained when it was individually expressed, PB2-encoding DNA reduces this value to 80% and mutant PA increases CKIIα′ accumulation to 300%. We do not know the meaning of the effect of mutated PA on CKIIα′, levels and further characterization should be carried out. These results clearly indicate that the PA effect is a general one and that it is not restrained to influenza virus proteins.

The PA-mediated effect is independent of the cell line used. We next examined whether the observed PA effect on protein accumulation could be the consequence of a general biological

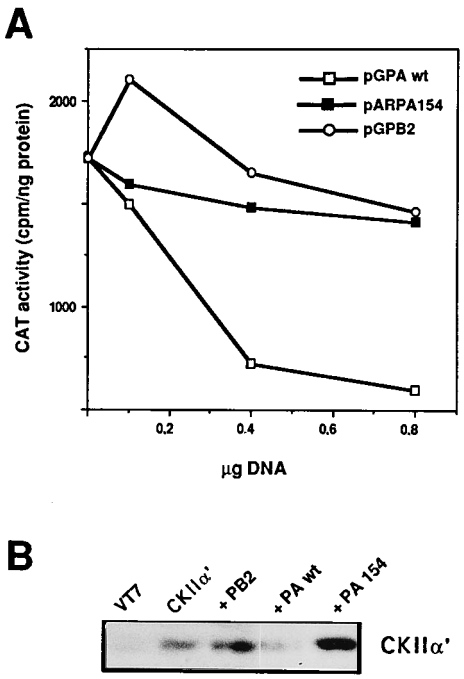

FIG. 3. Decrease of non-influenza virus protein accumulation upon PA coexpression. (A) T7-mediated CAT expression. Monolayers of VTF7-3-infected COS-1 cells were transfected with plasmid pT7 EMC-CAT (0.2 μg) either alone or with increasing amounts (0.1, 0.5, and 0.8 μg) of plasmid pGPB2, pGPA wt (wild type), or pARPA154. At 16 h posttransfection, cell extracts were prepared and tested for CAT activity. (B) Accumulation levels of CKIIα′ coexpressed with PA. COS-1 cells were infected with VTF7-3 and transfected with 2 μg of plasmid pBSHT-9.1, either alone or with 2 μg of the indicated plasmids. At 16 h posttransfection, cell extracts were prepared and analyzed by Western blotting with an anti-CKII-specific antibody.

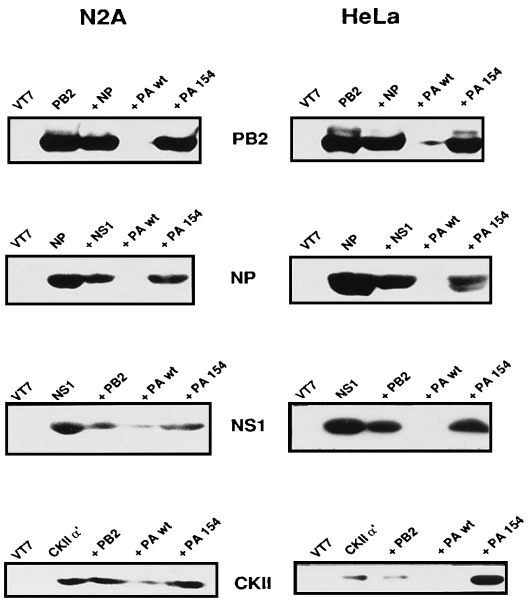

FIG. 4. PA protein effect is independent of the cell type. Subconfluent monolayers of either N2A or HeLa cells were infected with VTF7-3 and transfected with 0.25 μg of the corresponding reporter plasmid (pGPB2, pGNP, pGNS1, or pBSHT-9.1) alone or mixed with 0.25 μg of the indicated plasmids. Cell extracts were analyzed by Western blotting against the proteins indicated. wt, wild type.

activity. To that end, human epithelial-like cells (HeLa) and mouse neuroblastoma cells (N2A) were used as recipient cells for transfection experiments. The action of wild-type or mutated PA proteins was checked for PB2, NP, and NS1 influenza virus proteins as well as for the human α′ catalytic subunit of CKII, which is not expressed in these two types of cells. Several combinations of reporter plasmids were cotransfected and used as controls. The accumulation levels of the proteins encoded by all plasmids used were similar. The results (Fig. 4) indicate that wild-type PA but not mutated PA or the control plasmids used induced a reduction in the steady-state levels of all coexpressed proteins in these cell lines, although quantitative differences can be observed. Quantification of the Western blots showed that mutated PA and control plasmids produced variations of the coexpressed proteins ranging from 70 to 120% of the level obtained for the individual expression of the reporter gene. On the contrary, only 5 to 10% of the reporter protein remained when PA was coexpressed. These results suggest that the effect mediated by PA protein is exerted either directly or through an intermediate that is evolutionary conserved among the cell lines used in these experiments.

PA protein expression induces the degradation of coexpressed proteins. Our previous results showed that the effect of PA on the accumulation levels of coexpressed proteins was independent of the expression system chosen. Because in the two expression systems used, two different polymerases were responsible for the expression of the foreign genes, it seemed that the PA effect was not the consequence of changes at the transcriptional level and therefore PA could be acting at translational or posttranslational level. To answer this question, we carried out pulse-chase experiments in which PB2 and PA, encoded by either wild-type or PA154 mutant genes, were coexpressed by the vaccinia virus-T7 system. Singly or doubly transfected cells were either pulsed with [^{35}S]Met-Cys or pulsed and chased with an excess of unlabelled amino acids. The amount of labelled PB2 protein was determined by autoradiography after immunoprecipitation, and its stationary levels were determined by Coomassie blue staining of the immunoprecipitated samples. The results are shown in Fig. 5. We first observed that PB2 synthesis was similar or only slightly lower when this protein was coexpressed with wild-type PA. This small decrease, when it appears, might be due to a decrease in bacteriophage T7 RNA polymerase levels in the presence of wild-type PA, as determined by Western blot analysis (data not shown) (Fig. 5, pulse lanes). This result suggests that PA gene expression does not affect the accumulation of PB2 mRNA and does not alter the efficiency of its translation. On the other hand, the stability of PB2 protein was severely

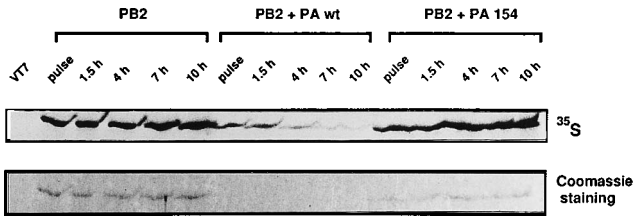

FIG. 5. PA protein expression reduces the half-life of coexpressed PB2. VTF7-3-infected COS-1 cells were transfected with 0.6 μg of plasmid pGPB2 and 0.12 μg of either pGPA wt (wild type) or pARPA154 as indicated. At 16 h posttransfection, cells were labelled for 15 min with a mixture of [^{35}S]Met and [^{35}S]Cys (700 μCi/ml) and lysed in RIPA buffer immediately (pulses) or after incubation for the indicated chase times. Cell extracts were used for immunoprecipitation of PB2 protein as described in Materials and Methods. After staining with Coomassie brilliant blue, the gel was dried and exposed.

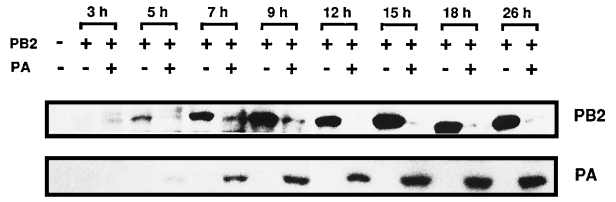

FIG. 6. Kinetics of appearance of the PA effect. COS-1 cells infected with VTF7-3 were mock transfected or transfected with 0.5 μg of plasmid pGPB2 either alone or with 0.5 μg of pGPB2 plus 0.5 μg of pGPA. At the indicated times posttransfection, cells were lysed in electrophoresis buffer, proteins were resolved by SDS-PAGE, and Western blots were developed with anti-PB2 and anti-PA antibodies.

diminished by coexpression with wild-type PA protein, as indicated by the reduction of labelled PB2 obtained after different chase periods. Again, this effect was not observed with mutant PA protein. The steady-state level of PB2, measured by Coomassie blue staining, showed a clear decrease specifically when coexpression was carried out with wild-type PA protein. The data obtained in this pulse-chase experiment indicate that PA induces a reduction in the stability of the coexpressed proteins and further reinforce the requirement for an active PA for this effect to occur.

PA-induced protein degradation is fast and does not require continuous protein synthesis. To further characterize the biological effect of PA, we measured the kinetics of appearance of the PA effect on protein degradation and the possible requirement of continuous protein synthesis. COS-1 cells were infected with VTF7-3 virus and transfected with pGPB2 either alone or in combination with pGPA. At several times after transfection, the accumulation levels of PB2 and PA proteins were determined by Western blot analysis. The results are shown in Fig. 6. The effect of PA protein on the accumulation of PB2 protein was observed at the earliest time at which PA protein was detectable, i.e., at 5 h after transfection. This biological effect was larger as incubation proceeded, in parallel with the progressive accumulation of PA protein, up to a maximum at 15 h posttransfection. These results indicate that a large accumulation of PA protein is not required for its biological effect to take place.

Finally, we studied whether the biological activity of PA protein requires continuous protein synthesis. To that end, additional pulse-chase experiments were carried out. The experimental design was identical to that described above, but cycloheximide was added during the chase periods in order to inhibit new protein synthesis. The results (Fig. 7) show that the increase in PB2 degradation as the consequence of PA expression is not abolished by inhibition of new protein synthesis. These results indicate that PA biological effect could be the consequence of either a direct effect of PA on protein degradation or the induction of a long-lived degradation system.

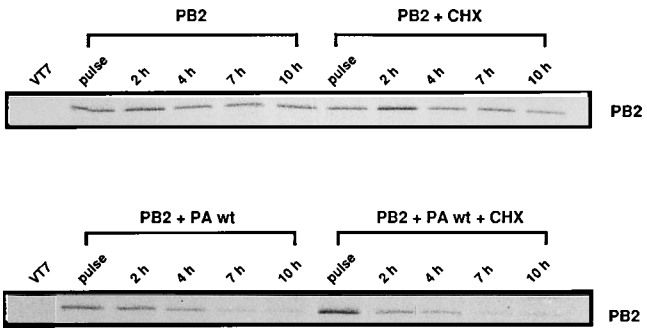

FIG. 7. PA-induced protein degradation does not require continuous protein synthesis. COS-1 cells infected and transfected as described for Fig. 6 (pGPB2 alone or pGPB2 plus pGPA) were labelled at 16 h posttransfection with a mixture of [^{35}S]Met and [^{35}S]Cys (20 μCi/ml) and chased without or with 100 μg of cycloheximide (CHX) per ml. At the indicated times, the cells were lysed in RIPA buffer and the cell extracts were used to immunoprecipitate PB2 protein. After SDS-PAGE, the gel was exposed to visualize the radioactive bands. wt, wild type.

DISCUSSION

The results presented in this report indicate that the individual expression of the influenza virus PA polymerase protein induces a general decrease on the steady-state levels of proteins coexpressed with this viral polymerase subunit. This reduction is independent of (i) the influenza virus or non-influenza virus origin of the proteins (Fig. 1 and 3), (ii) the system used for coexpression (Fig. 2), and (iii) the type of recipient cell in which the expression of the foreign genes is carried out (Fig. 4). The specificity of this biological activity was tested by using a mutant PA gene (PA154) that is completely inactive in the in vivo-reconstituted polymerase CAT assay. Such a mutant did not show any alteration in the level of accumulation of coexpressed proteins (Fig. 2 to 4). These results indicate that a functional PA protein is required for the inhibitory effect to take place and furthermore make it very unlikely that this effect was the result of a competition for the genes coexpressed at either the transcriptional or translational level. Further characterization of the mechanism involved in the PA effect showed that it is not the consequence of changes in protein synthesis (Fig. 5), suggesting that variations at the mRNA levels, like variations in transcription or stability, should not be responsible. In this context it should be mentioned that quantification of PB2 RNA from cells transfected individually with a PB2-encoding plasmid or together with a wild-type PA-encoding plasmid did not show any difference (data not shown). By contrast, pulse-chase experiments indicated that PA activity produces a clear reduction of the half-life of the coexpressed proteins. These results indicate that PA protein leads to a stimulation of protein degradation in the cell. To the best of our knowledge, this is the first description of a viral gene, not known to encode a protease, that induces a generalized protein degradation in the expressing cell.

No sequence motifs from the Prosite data bank could be identified in the PA protein. In particular, no similarities with known protease sequences could be detected. Although PA protein contains a sequence similar to that of the consensus A site of ATP-binding proteins (9), this is not conserved among PA proteins from different influenza viruses. In view of this lack of predictive information, the biological effect of PA protein action has been characterized in regard to its kinetics. No delay could be observed between the detection of PA protein and the decrease of coexpressed PB2 protein accumulation (Fig. 6), indicating that a previous accumulation of PA to attain a significant threshold is not necessary. The effect of PA expression was observed in cell lines differing in origin and differentiation state, although quantitative differences could be observed between them (Fig. 1 and 4). The fact that the PA protein biological effect does not require continuous protein synthesis once the protein has been expressed (Fig. 7) is compatible with a direct action of PA. However, all previous experiments are also compatible with the possibility that PA can act in a "hit-and-run" fashion, activating a long-lived cellular protein degradation pathway that would remain in an active state for longer periods of time. Because PA is also able to induce its own degradation (data not shown), an autoproteo-

lytic activity should be predicted if the observed general degradation of cellular proteins is the direct consequence of the presence of PA.

Study of the functionality of the polymerase subunits has proved to be a difficult task, particularly in the case of PA protein. In this paper, we report the first activity that can be assigned to this subunit, i.e., a direct or indirect induction of protein degradation. PA protein effect can also be observed when PA is being synthesized together with the other components of the RNP (PB1, PB2, and NP) and, furthermore, in the presence of a virus-like CAT RNA (Fig. 1). This finding suggests that the effect on protein stability could be carried out even when PA is a component of an active RNP complex. However, it is also possible that the detected PA activity was the consequence of an overexpression of PA over the stoichiometric requirements for the formation of a functional viral core.

Is the PA function described in this report relevant in a virus-infected cell? In the course of an influenza virus infection, a decrease in cellular mRNA synthesis, probably due to the virus-induced cap-snatching activity, has been described, as well as an inhibition of nucleocytoplasmic transport of the cellular mRNAs and/or a degradation of cellular RNAs in the nucleus (17). The cellular cytoplasmic mRNAs are degraded during the infection (3, 15), and an inhibition of cellular protein synthesis has also been reported, even in the absence of substantial cytoplasmic mRNA degradation (17). However, no evidence has been provided regarding a generalized proteolysis during influenza virus infection. Since the experiments described here were carried out by expression of influenza virus genes from cloned DNA, they do not provide clues about the situation in the infected cell. However, our results indicate a high susceptibility of other viral proteins to the action induced by PA gene expression. If this action represents a biological PA effect, its expression should be blocked in the course of a normal virus infection. In this regard, it is worth mentioning that the transport of PA protein to the nucleus is delayed in the infection compared with the transport of the other RNA polymerase components (1, 28) and that the nuclear localization of PA protein, which has its own nuclear localization signal (27), is enhanced by coexpression of other viral RNA polymerase subunits (28). This interpretation is in line with the fact that PA154 mutant protein is exclusively cytoplasmic (27) and involves the suggestion that for the induction of protein degradation to occur, PA protein has to be localized to the nucleus of the transfected cell. Experiments are in progress for the phenotypic analysis of PA mutants.

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