JVI Journals.ASM.org

CHD6, a Cellular Repressor of Influenza Virus Replication, Is Degraded in Human Alveolar Epithelial Cells and Mice Lungs during Infection

Roberto Alfonso,[a,b]* Ariel Rodriguez,[a,b] Paloma Rodriguez,[a,b] Thomas Lutz,[a,b]* Amelia Nieto[a,b]

Centro Nacional de Biotecnología (CNB-CSIC), Darwin 3, Cantoblanco, Madrid, Spain[a]; Ciber de Enfermedades Respiratorias, Mallorca, Illes Balears, Spain[b]

The influenza virus polymerase associates to an important number of transcription-related proteins, including the largest subunit of the RNA polymerase II complex (RNAP II). Despite this association, degradation of the RNAP II takes place in the infected cells once viral transcription is completed. We have previously shown that the chromatin remodeler CHD6 protein interacts with the influenza virus polymerase complex, represses viral replication, and relocalizes to inactive chromatin during influenza virus infection. In this paper, we report that CHD6 acts as a negative modulator of the influenza virus polymerase activity and is also subjected to degradation through a process that includes the following characteristics: (i) the cellular proteasome is not implicated, (ii) the sole expression of the three viral polymerase subunits from its cloned cDNAs is sufficient to induce proteolysis, and (iii) degradation is also observed *in vivo* in lungs of infected mice and correlates with the increase of viral titers in the lungs. Collectively, the data indicate that CHD6 degradation is a general effect exerted by influenza A viruses and suggest that this viral repressor may play an important inhibitory role since degradation and accumulation into inactive chromatin occur during the infection.

The influenza virus contains a segmented genome of eight negative-sense and single-stranded RNA molecules, whose expression takes place in the nucleus of the infected cell. Genomic RNAs (vRNAs) form ribonucleoprotein complexes (vRNPs) that are constituted by the three subunits of the polymerase (PB1, PB2, and PA) and the nucleoprotein (NP), which are responsible for genome expression (1–5). For viral replication, the vRNAs are copied to form full-length positive-stranded RNAs (cRNAs), which serve as templates for vRNA synthesis. During transcription, capped and polyadenylated viral mRNAs are synthesized by the viral polymerase. The mRNA synthesis is primed by short-capped oligonucleotides of around 10 to 12 nucleotides scavenged from *de novo* synthesized host cell pre-mRNAs by a viral endonuclease activity (6). This transcription strategy involves a functional coupling between viral and cellular transcription for the cap-snatching process, but this functional association is broader and applies to different steps of viral mRNA metabolism. Indeed, two of the viral transcripts are spliced (7, 8), but the influenza virus does not possess a viral splicing system, since it is dependent on the host splicing machinery (9), an activity related to the RNA polymerase II (RNAP II) transcription. Furthermore, the influenza virus uses the mRNA export machinery of the infected cell at least for some of its mRNAs, and an active RNA polymerase II is required to facilitate nuclear export of selected viral mRNAs (10). In agreement with this transcriptional association, interaction of the viral polymerase with host cell transcription-related factors has been reported, among which the interaction with the largest subunit of the RNAP II (11) should be emphasized. Other transcription-related factors found to interact with the viral polymerase are Erb-B3 binding protein 1 (Ebp-1) (12), which represses transcription of cell cycle genes regulated by E2F transcription factors (13); DDX5 protein (14), a transcription coactivator that may play a role in transcription initiation (15); SFPQ/PSF factor (14), which stimulates pre-mRNA processing (16) and is essential for influenza virus transcription increasing the efficiency of viral mRNA polyadenylation (17); and hCLE, a positive modulator of the RNAP II (18, 19) which is required for influenza virus replication (20).

The eukaryotic DNA is packaged in a higher-order structure known as chromatin, and chromatin remodelers play a critical role in allowing access to the transcription machinery to chromatin regions. Chromodomain-helicase DNA binding proteins (CHD) are a family of chromatin remodelers constituted by three different subfamilies that contribute to the dynamics of chromatin structure, affecting the binding of transcription factors and, therefore, modulating the initiation and elongation steps of transcription (21–23). The CHD6 protein belongs to subfamily III of the CHD family and is a transcription-related factor since it colocalizes with RNAP II and is present at sites of mRNA synthesis (24). In addition, we previously observed that CHD6 negatively modulated influenza virus replication, showing for the first time the importance of a protein that modifies chromatin in the life cycle of this virus (25). In fact, influenza virus infection induces marked remodeling of the host nuclear architecture, and accordingly it has been described that viral ribonucleoproteins are closely bound to the nuclear matrix or to chromatin components (26–29). This binding may be mediated, at least in part, through interaction of NP with nucleosomes, since NP interacts with histone tails *in vitro* (30). In agreement with this association, it has been

Received 2 March 2012 Accepted 25 January 2013
Published ahead of print 13 February 2013
Address correspondence to Amelia Nieto, anieto@cnb.csic.es.
* Present address: Robert Alfonso, Laboratory of Viral Diseases, NIAID National Institutes of Health, Bethesda, Maryland, USA; Thomas Lutz, Dr. Knoell Consult GmbH, Mannheim, Germany.

doi:10.1128/JVI.00554-12

proposed that viral transcription and replication take place in DNase insensitive nuclear fractions that include chromatin and/or cellular matrix (31). We have previously described the interaction of the PA polymerase subunit and viral polymerase complex with CHD6 (18), which relocates to inactive chromatin upon infection and negatively modulates influenza virus replication (25). Despite the described coupling between viral and cellular transcription, previous reports have shown that influenza virus infection causes the degradation of RNAP II, with a concomitant inhibition of cellular mRNA synthesis (32–34). Interestingly, RNAP II proteolysis is not observed in cells infected with the A/PR/8/34 (PR8) and A/Ann Arbor/6/60 cold-adapted (AA*ca*) attenuated viral strains, highlighting the possibility that a link between this phenomenon and viral pathogenicity may exist (32). Two main questions are studied in this paper: (i) does viral polymerase activity depend on the chromatin remodeler CHD6 and (ii) is CHD6, like RNAPII, a degradation target during viral infection? We show here that CHD6 serves as a negative modulator of the influenza virus polymerase transcription and replication and undergoes proteolytic degradation during infection in cultured cells and in an animal model. Moreover, degradation is not dependent on the cellular proteasome pathway and can be observed in cells that express only viral polymerase.

MATERIALS AND METHODS

Biological materials. The cell lines used in this study were HEK293T and A549. The influenza viruses A/Victoria/3/75 (H3N2) (VIC), A/WSN/33 (H1N1) (WSN), A/PR8/8/34 (H1N1) (PR8), A/New Caledonia/20/99 (H1N1) (NC), A/England/1/51 (H1N1) (E), A/Wyoming/3/2003 (H3N2) (Wy), A/Ann Arbor/6/60 (H2N2) (AA), A/Ann Arbor/6/60 cold-adapted (H2N2) (AA*ca*), A/Turkey/Wisconsin/66 (H9N2), A/California/07/09 (CAL), and a recombinant influenza virus containing the RNP genes from the VIC strain and all other genes from the WSN strain (35) were used. Vesicular stomatitis virus (VSV) of the New Jersey serotype was a gift from M. Esteban, and P. Fortes kindly provided adenovirus serotype 5 (AdV). The plasmids expressing the influenza virus polymerase subunits from the AA and AA*ca* strains were generously distributed by K. Subbarao. The plasmids pCMVPA, pCMVPB1, and pCMVPB2 have previously been described (36). Proteasome inhibitor MG132 and cycloheximide were from Sigma. The protease inhibitor Complete was from Roche.

Virus infection. Cells were infected with the influenza virus at a multiplicity of infection (MOI) of 3 PFU/cell and with VSV and AdV at MOIs of 5 and 7.5 PFU/cell, respectively. At different hours postinfection (hpi), the cells were collected in phosphate-buffered saline (PBS) with protease (Complete) inhibitors, and the cell pellet was resuspended in Laemmli buffer (37).

Cell transfection. Cell cultures were transfected by the calcium-phosphate method (38) with pCMV plasmids expressing PB1, PB2, or PA from the VIC strain or with pBD plasmids expressing the polymerase subunits from the AA and AA*ca* strains. After 24 h of transfection, the cells were collected and used for Western blot analysis.

Western blotting. Western blotting was carried out as described previously (33). The following primary antibodies were used: for CHD6, an immunopurified rabbit antibody (1:1,000) from Bethyl Laboratories; for CHD3, a rabbit polyclonal antibody (1:300) from Transduction Laboratories; for CHD7, a rabbit polyclonal antibody (1:500) from Abcam; for CHD1, a rabbit anti-human antibody (1:1,000) from Cell Signaling; for RNA polymerase II, monoclonal antibody 8WG16 (1:500) from BabCo; for PA, monoclonal antibody 14 (1:250) (39); for PB2, monoclonal antibody 22 (1:25) (39); for PB1, a rabbit polyclonal antibody (1:1,000) (40); for NP, a rabbit polyclonal antibody (1:5,000) (41); for VSV detection, a monoclonal antibody mix against G, N, and M viral proteins provided by M. Esteban; for DDX5 detection, a goat anti-human antibody from Abcam (1:1,000); and for β-tubulin, a mouse monoclonal antibody (1:15,000) from Sigma.

Quantitative reverse transcription-PCR (qRT-PCR) detection. Total RNA was isolated using TRI reagent (Sigma) by following the manufacturer's indications. For CHD6 and β-actin mRNA detection, which was used for standardization, reverse transcription with the applied high-capacity cDNA reverse transcription kit was used. For viral RNA detection, hot-start reverse transcription with a tagged primer was used (42). cDNAs complementary to the three types of influenza virus RNA for segment five were synthesized using primers with an additional 18 to 20 unrelated nucleotide tags at the 5′ end as described previously (42). The following oligonucleotides were used: for the RT of the vRNA, 5′-GGCCGTCATGGTGGCGAATTGCCTGCCTGTGTGTATGGA-3′; for the cRNA, 5′-GCTAGCTTCAGCTAGGCATCGTAGAAACAAGGGTATTTTTCC-3′; and for the mRNA, 5′-CCAGATCGTTCGAGTCGTTTTTTTTTTTTTTTTAATTGTCGTAC-3′.

For the detection of mRNA levels, quantitative real-time PCR (qPCR) was performed with Power SYBR green PCR master mix from Applied Biosystems on an Applied Biosystems 7500 real-time PCR system. Four microliters of the cDNA was added to the qPCR mixture (10 μl Power SYBR green PCR master mix from Applied Biosystems [2×], 1.5 μl forward primer [10 μM], 1.5 μl reverse primer [10 μM], 3 μl double-distilled water). The cycle conditions of qPCR were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Data were analyzed with 7500 software version 2.0.5, and each sample was normalized based on the amount of β-actin. The following oligonucleotides were used: for CHD6, 5′-TAAGCCAGATTCAGACCGCT-3′ and 5′-TGAGCCTGCAAGTCATTTTG-3′; for β-actin, 5′-CCCAGCACAATGAAGATCAA-3′ and 5′-CGATCCACACGGAGTACTTG-3′; for the vRNA, 5′-GGCCGTCATGGTGGCGAAT-3′ and 5′-TCCACACCAGCTGACTCTTG-3′; for the cRNA, 5′-GCTAGCTTCAGCTAGGCATC-3′ and GAGCTCTCGGACGAAAAGG; and for the mRNA, 5′-CCAGATCGTTCGAGTCGT-3′ and 5′-GAGCTCTCGGACGAAAAGG-3′.

Mice. Twenty-two 6-week-old BALB/c mice were infected intranasally with 5×10^5 PFU of a recombinant influenza virus containing RNP genes from the VIC strain and all other genes from the WSN strain (35), and four mice were mock infected. Their body weights were measured daily, and they were sacrificed and their lungs excised at different days after inoculation. The lungs were homogenized in PBS-0.3% bovine serum albumin (BSA) in a Dounce homogenizer and centrifuged at 10,000 × *g*. The viral titers were measured in the supernatants by plaque assay. The same lung preparations were used for Western assays.

RESULTS

CHD6 silencing increases influenza virus RNA replication and transcription. We previously found that CHD6 interacts with the viral polymerase complex during infection and negatively modulates influenza virus growth (25). Next, we asked whether CHD6 could regulate the influenza virus polymerase activity, which could account for the observed increase in viral particle production. In order to answer this question, stable A549 cells individually expressing either a control short hairpin RNA (shRNA) (TM) or two CHD6 shRNAs with different silencing efficiencies (shCHD6.1 and shCHD6.2) (25) were left uninfected or infected with the influenza virus VIC strain at an MOI of 3. Ten hours later, samples for each cell type and condition were taken and used for RT-PCR detection of CHD6 mRNAs and viral RNAs (vRNA, cRNA, and mRNA). A previously described strand-specific quantitative real-time RT-PCR procedure for distinguishing influenza vRNA, cRNA, and mRNA species on infected cells (42) was used as detailed in Materials and Methods. Cellular extracts were also prepared to detect CHD6, viral (PB1 and NP), and control (β-tubulin) proteins by Western blotting. A reduction in CHD6 protein levels was found in control (TM) infected cells compared to

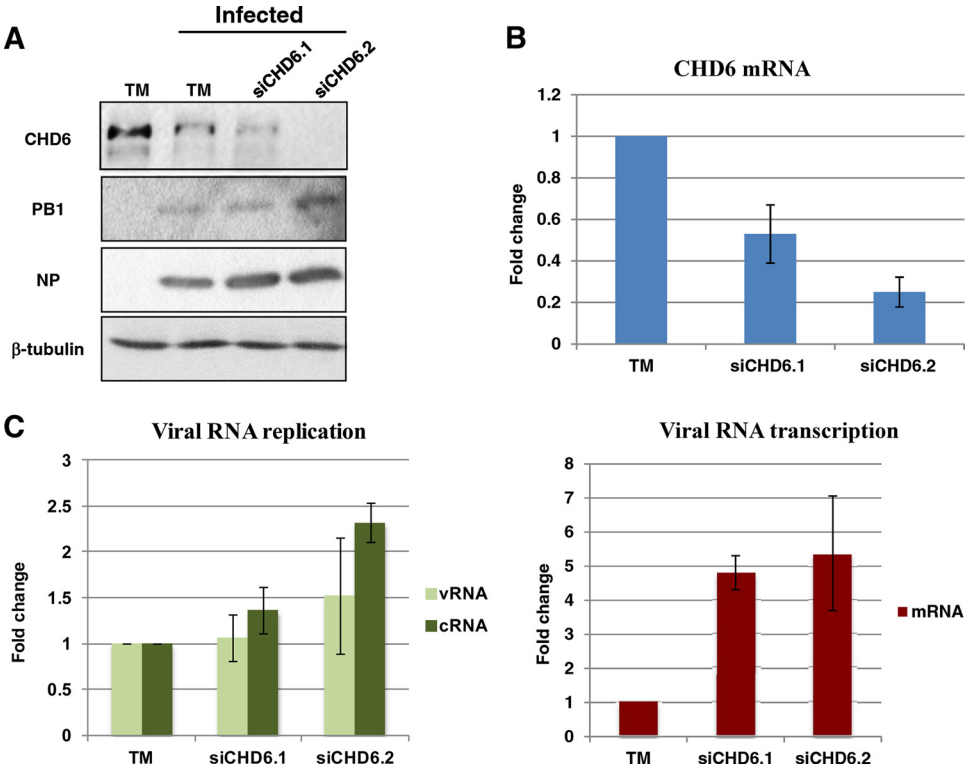

FIG 1 CHD6 silencing increases influenza virus RNA replication and transcription. (A) Stable A549 cells expressing a control silencer (TM) or specific silencers for CHD6 (siCHD6.1 and CHD6.2) were left uninfected or infected at an MOI of 3. At 10 hpi, samples were taken and used for Western blotting against the indicated proteins. A representative experiment is presented. (B and C) Total RNAs from stable A549-infected cells as described in panel A were isolated and used for qRT-PCR detection of CHD6 mRNAs (B) or influenza virus RNAs (C). Two experiments were performed with three biological replicates. The results represent the means ± standard deviations (SD) from the two experiments.

that of uninfected cells (see below). Within the infected cells, decreased accumulation of CHD6 protein levels were found in the CHD6 shRNA stable cell lines. This reduction was higher in the shCHD6.2-expressing cells (as previously reported [25]), while β-tubulin levels did not show variations (Fig. 1A). PB1 and NP accumulation was observed in every condition, indicating that influenza virus infection took place (Fig. 1A). Accordingly, reduction of CHD6 mRNA levels was observed in CHD6-silenced cells, and there was an even greater reduction in CHD6 mRNA levels in siCHD.2 cells (Fig. 1B). A higher production of viral RNAs corresponding to RNA replication (vRNAs, cRNA) and transcription (mRNAs) was found in CHD6 silenced cells, being higher in cells expressing the siCHD6.2 silencer (Fig. 1C). It should be highlighted that silencing of CHD6 produces a higher increase in viral transcription than in viral replication, suggesting that CHD6 targets mainly the transcription step of viral expression. No significant changes were observed in β-actin RNAs in any situation (data not shown), and its levels were used to normalize the production of viral RNAs. These results indicate that CHD6 negatively modulates both influenza virus RNA replication and transcription and agree with previous data showing an increase of viral titer in CHD6-silenced cells (25). Moreover, the increase in viral RNA production correlates with the degree of CHD6 silencing (Fig. 1A and B) and the viral titer (25), suggesting that viral polymerase plays a key role in the CHD6-mediated regulation of the influenza virus life cycle. We have also explored the effect that the overexpression of CHD6 has on influenza virus replication. Infected cells overexpressing a hemagglutinin (HA)-tagged CHD6 protein produce viral titers similar to those obtained in control cells (data not shown). These results could indicate that the amount of endogenous protein is sufficient to inhibit viral replication, and larger amounts do not cause a larger effect.

Influenza virus infection induces degradation of the CHD6 chromatin remodeler independently of the proteasome pathway. CHD6 protein levels were reduced in the above-described experiment in cells infected with influenza virus compared to those in mock-infected cells (see Fig. 1A). As mentioned, RNAP II is degraded during influenza virus infection in a process where the viral polymerase plays a major role. Taking into account that CHD6 is a viral polymerase-associated factor that negatively modulates viral polymerase transcription and replication, we examined whether CHD6 becomes degraded upon infection. With this aim, human alveolar epithelial cells (A549) were infected with the VIC strain, and at different hpi, total cell extracts were used for Western blot analysis with antibodies that recognize CHD6. RNAP II accumulation was simultaneously observed to monitor its degradation during influenza virus infection. The accumulation levels of other chromatin remodelers belonging to the three different CHD subfamilies were also evaluated. We chose CHD1 of subfamily I, CHD3 of subfamily II, and CHD7 belonging to subfamily III, as does CHD6. To assess the progression of the infection, the amounts of PB1 and PA polymerase subunits were analyzed as well as that of β-tubulin. The results (Fig. 2A) indicated that the accumulation of CHD6 decreases at 6 hpi, reaching

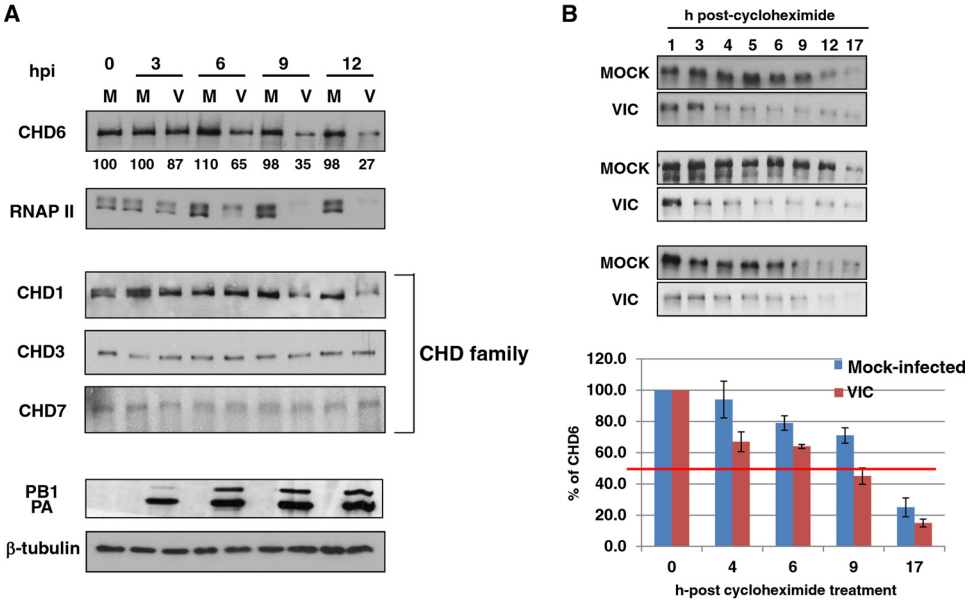

FIG 2 Influenza virus infection induces degradation of CHD6 chromatin remodeler. (A) A549 cells were infected with the influenza virus VIC strain, and at the designated hpi, CHD6 and the indicated proteins were monitored in total cell extracts by Western blotting. Five different experiments were carried out, and a representative experiment is shown. Quantification of the amount of CHD6 during the virus infection is shown at the bottom of the CHD6 panel. (B) Half-life of CHD6 in mock-infected and influenza virus-infected cells. A549 cells were mock infected or infected with the VIC strain of influenza virus and treated with cycloheximide (100 μg/ml) at 6 hpi. At the indicated times after treatment, aliquots were analyzed for Western blotting to detect CHD6. Three different experiments were performed, and in every case the accumulation levels of CHD6 both in mock-infected and infected cells at 6 h were considered 100%.

low levels at later times postinfection (12 h). The amounts of CHD3 and CHD7 did not show variations as the infection progressed. CHD1 was also degraded during the infection with a slightly delayed kinetic. The degradation of CHD6 and CHD1, but not of other CHD members, suggests a complex interrelation between the influenza virus infection and specific members of this family of ATP-dependent chromatin remodelers.

It is well established that influenza virus infection shuts down host cell protein synthesis (43, 44). Inhibition of cell host transcription and host mRNA degradation take place during infection (33, 45, 46) and definitely contribute to the virus-induced shutoff. Therefore, determination of the amount of CHD6 mRNA during infection would not provide insights into the mechanism involved in the observed decrease of CHD6 protein levels. To distinguish between an effect caused by the general virus-induced cellular shutoff and a specific degradation of CHD6 during infection, we studied the half-life of CHD6 in mock-infected and infected cells. Thus, mock-infected or infected A549 cells were treated with cycloheximide at 6 hpi to stop *de novo* protein synthesis. At different times after treatment, cell extracts were taken and used for Western blot analysis against CHD6. The experiment was repeated three times, and in every case the accumulated CHD6 protein just before the addition of the drug either in mock-infected or infected cells was considered 100%. The estimated half-life of CHD6 was around 12 h in mock-infected cells and around 8 h in infected cells (Fig. 2B). Since the half-life of CHD6 was reduced during infection, this result indicates that influenza virus infection triggers CHD6 degradation.

Next we tested the involvement of the proteasome pathway in CHD6 degradation. Cultured A549 cells were infected with the VIC strain or remained uninfected in the presence or absence of the proteasome inhibitor MG132, which was added 1 h before infection. At different hpi, the accumulation levels of the PA polymerase subunit and CHD6 were analyzed (Fig. 3A). Almost total degradation of CHD6 was observed at 12 hpi, irrespective of the presence of the proteasome inhibitor. As a control of the effectiveness of the drug, the extracts were probed with antibodies specific for ubiquitin, and a significant accumulation of ubiquitinated proteins was observed upon MG132 treatment (Fig. 3B). These results suggest that the ubiquitin-mediated proteasomal degradation pathway does not mediate CHD6 degradation triggered by influenza virus infection.

Degradation of CHD6 is a general feature triggered by influenza A viruses. To determine whether degradation of CHD6 is a general feature induced by influenza A viruses, infections with another laboratory-passaged influenza strain and several natural

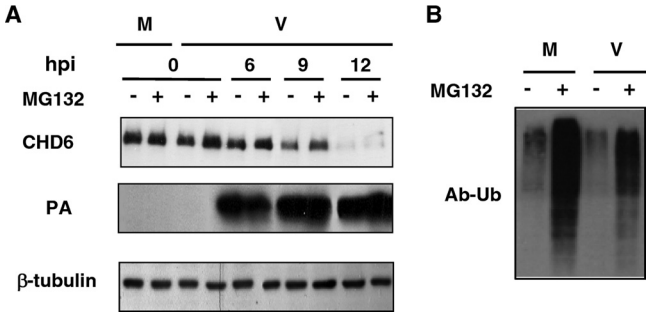

FIG 3 Proteasomal degradation pathway does not mediate CHD6 degradation during influenza virus infection. (A) A549 cells, treated (+) or not treated (−) with 25 μM MG132, were mock infected (M) or infected with the VIC strain (V). At the indicated hpi, CHD6 and the designated proteins were detected by Western blotting. (B) Western blot using anti-ubiquitin antibody of mock-infected and infected cells at 12 hpi.

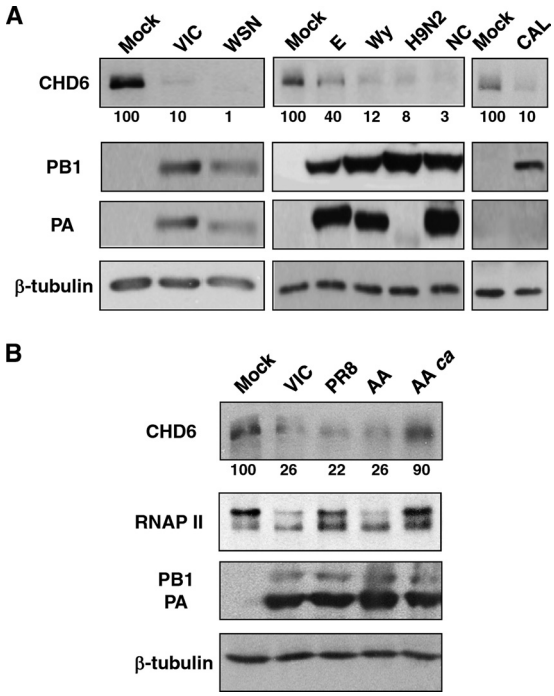

FIG 4 Degradation of CHD6 is a general feature triggered by influenza A viruses. (A) A549 cells were mock infected or infected with the VIC, WSN, E, Wy, H9N2, NC, or CAL strains at 3 PFU/cell. At 15 hpi, CHD6, PA, PB1, and β-tubulin proteins were monitored by Western blotting of the total cell extracts. (B) A549 cells were mock infected or infected with the PR8, VIC, AA, or AA*ca* strains of the influenza virus at 3 PFU/cell at 32°C. At 24 hpi, CHD6, RNAP II, and the indicated proteins were monitored by Western blotting of total cell extracts. Quantification of the amount of CHD6 is shown at the bottom of the CHD6 panels.

isolates were carried out. Cultured A549 cells were infected with A/WSN/33 (WSN) and with different human isolates of the H1N1 subtype, such as A/England/1/51 (E), A/New Caledonia/20/99 (NC), and the 2009 pandemic strain A/California/07/09 (CAL), or the H3N2 subtype, such as A/Wyoming/3/2003 (Wy). An avian strain, A/Turkey/Wisconsin/66 of the H9N2 subtype (H9N2), was also used. In all cases, the accumulation levels of CHD6, PB1, PA, and β-tubulin at 15 hpi were examined (Fig. 4A). As previously described (32), the detection of PA from the H9N2 and CAL strains was almost undetectable, probably due to the absence of the corresponding epitope recognized by the monoclonal antibody in the avian proteins. Nevertheless, a successful monitoring of infection by these strains through PB1 accumulation was possible. All the viruses produced CHD6 degradation, supporting the notion that this process is a general feature of influenza A viruses.

Attenuated influenza viruses are used to produce reassortant vaccine strains containing the six internal gene protein segments of these attenuated viruses and the NA and HA segments of the circulating viruses. Two main attenuated strains are currently used in the generation of vaccines, the A/PR/8/34 (PR8) and the A/Ann Arbor/6/60 cold-adapted (AA*ca*) viruses. The PR8 virus is attenuated in humans (47) and chickens (48), and it has been used to produce inactivated influenza vaccines. On the other hand, the AA*ca* influenza virus is avirulent in humans as well as in animal models (mice and ferrets) (49). Moreover, this cold-adapted strain has been used to produce safe live influenza A virus vaccines (49, 50) that are being commercially developed for use in children.

As opposed to nonattenuated influenza viruses, the attenuated influenza strains PR8 and AA*ca* are not capable of inducing RNAP II degradation (32). Thus, we asked if CHD6 degradation occurs upon infection with these nonpathogenic strains. With this aim, A549 cells were infected with VIC, PR8, AA, or AA*ca* strains at 32°C, a permissive temperature for the AA*ca* virus. The accumulation levels of CHD6 and RNAP II were analyzed at 24 hpi by Western blotting. Accumulation of PB1 and PA were analyzed as a measure of the progression of the infection, and β-tubulin was used as the loading control. The results obtained (Fig. 4B) showed that during infection of A549 cells with the PR8 strain, CHD6 was degraded as efficiently as in the VIC infection. Similar results in CHD6 degradation were observed when PR8 infection was performed at 37°C (data not shown). In contrast, this effect was attenuated when the cells were infected with the AA*ca* strain, since only some CHD6 degradation was observed after 24 hpi. Moreover, CHD6 was degraded when the parental nonattenuated AA strain was used. Therefore, CHD6 would seem to be a very susceptible target for the virus-induced proteolysis since only a very attenuated strain, such as the AA*ca* virus, shows an impaired CHD6 degradation activity.

Specificity of influenza virus-induced CHD6 degradation. As reported, the influenza virus requires a close functional association with the cellular transcription apparatus and causes severe alterations in the host cell expression system. Since chromatin remodelers modulate cellular gene expression, we examined whether CHD6 degradation also took place upon infection with different viruses which impair the host-cell expression system or modify the nucleus of the infected cell. Two viruses that are human pathogens and present some similarities with the influenza virus were chosen. The first virus analyzed was vesicular stomatitis virus (VSV), a negative, single-stranded but nonsegmented RNA virus that forms ribonucleoproteins and whose life cycle takes place in the cytoplasm of the infected cell. Besides the similarity of its genome characteristics, VSV and the influenza virus share activation of the RIG-I RNA sensor during infection (51) and impairment of viral replication by the cellular Mx protein (52). Infection with VSV produces host cell transcription shutoff by inactivation of the host general transcription initiation factor TFIID (53). Moreover, VSV infection induces inhibition of DNA synthesis (54) and nuclear cytoplasmic transport as well as processing of host RNA (55, 56). Thus, cultured A549 cells were infected with VSV at a high MOI, and the accumulation levels of CHD6, viral proteins (G, N, and M), and β-tubulin were monitored at different hpi (Fig. 5A). Production of viral proteins was clearly visible at 4 hpi, and their accumulation increased during the infection. In this situation, CHD6 levels remained constant through the infection without any indication of degradation.

The other virus examined was adenovirus, which has a double-stranded DNA genome and strongly depends on the host cell transcription system. Similar to the influenza virus, adenovirus replicates in the nucleus of the infected cell, inducing important alterations in nuclear structures, such as the nuclear matrix (57) or the promyelocytic leukemia (PML) bodies (58). Infection with adenovirus serotype 5 was then performed in A549 cells at a high MOI, and cell extracts were obtained at different hpi to analyze CHD6 accumulation. As presented in Fig. 5B, the amount of CHD6 remained unaltered through the infection, which was monitored simultaneously by immunofluorescence assays.

Collectively, the results suggest that CHD6 degradation pro-

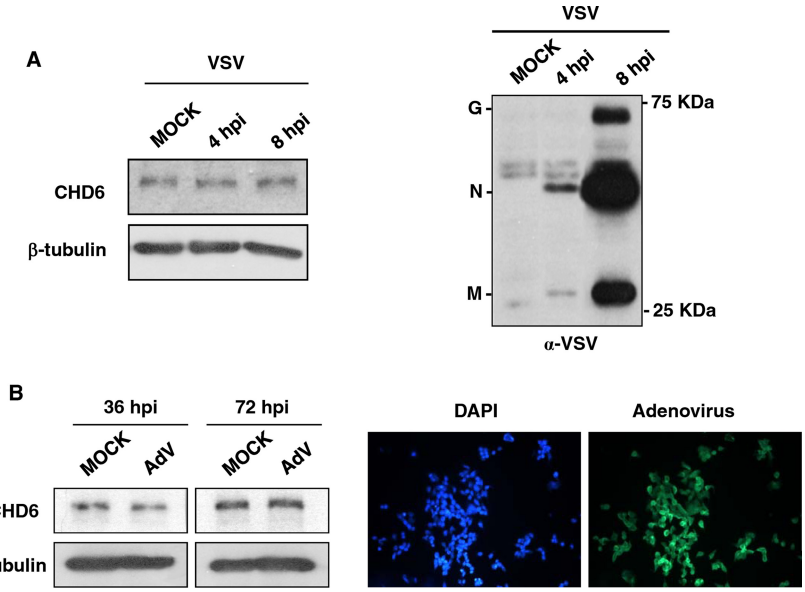

FIG 5 VSV and adenovirus infection do not trigger CHD6 degradation. (A) A549 cells were mock infected or infected with VSV at 5 PFU/cell. At different hpi, total cell extracts were processed for CHD6 and β-tubulin detection (left) or the indicated VSV proteins (right) by Western blotting. (B) A549 cells were mock infected or infected with the adenovirus serotype 5 at 7.5 PFU/cell. At the times indicated, CHD6 and β-tubulin accumulation was monitored by Western blotting (left). Right, DAPI staining and immunofluorescence analysis of the A549-infected cells at 36 hpi, using monoclonal antibody MAB805 (1:200) to detect adenovirus protein.

duced by influenza virus infection could be specific for this virus since other viruses having a similar genomic constitution, such as VSV, or causing alterations on subnuclear structures, such as adenovirus, do not trigger the degradation process.

The viral RNA polymerase complex is sufficient to induce the degradation of CHD6. Looking for viral proteins involved in RNAP II degradation, we had previously observed that neither the individual expression of polymerase subunits nor combinations of two of them induced proteolysis. In contrast, the coexpression of the three subunits triggered RNAP II degradation (33). Thus, we checked whether proteolysis of CHD6 occurs in the same conditions. Cultured HEK293T cells, which are very efficiently transfected, were then used for transfection with plasmids expressing individually PB1, PB2, or PA from the VIC strain, combinations of two of these plasmids, or coexpression of the three plasmids to reconstitute the viral polymerase. A total of 2 μg of plasmids was used in all cases. After 24 h of transfection, the amounts of CHD6 were analyzed by Western blotting. The individual expression of polymerase subunits or combinations of two of them did not induce significant degradation (Fig. 6A), whereas the *in vivo* reconstituted influenza virus polymerase triggered CHD6 proteolysis. In addition, a dose-dependent experiment using increasing amounts of plasmids expressing PA, PB1, and PB2 shows a positive correlation between CHD6 degradation and the levels of viral polymerase expression (Fig. 6B).

Since a decreased degradation capacity was observed in cultured cells infected with the AA*ca* strain (Fig. 4B), we tested the effect of the reconstituted viral polymerase from this strain on CHD6 degradation. Thus, HEK293T cells were left untransfected or transfected with plasmids expressing the three polymerase subunits from the VIC, AA, and AA*ca* strains at 32°C, and 24 h later, samples were used for CHD6, PB1, PA, PB2, and β-tubulin detection by Western blotting (Fig. 6C). The results indicated that reconstituted viral polymerases from VIC and AA strains produce CHD6 degradation, while the reconstituted polymerase from the AA*ca* strain does not significantly degrade CHD6, in agreement with the decreased capacity of the AA*ca* virus to induce the degradation process. A weak PB1 accumulation from the AA strains compared with that of the VIC strain was observed. Since PB1 accumulates properly in either AA- or AA*ca*-infected cells (Fig. 4B), the results obtained could represent PB1 instability when it is expressed out of the infection. Nevertheless, the results indicate that sufficient viral polymerase is reconstituted in cells transfected with the AA plasmids since CHD6 degradation occurs in cells expressing AA polymerase in contrast with cells expressing AA*ca* polymerase. These data provide evidence that viral polymerase expressed out of the context of infection is also capable of degrading CHD6 and, moreover, that a correlation exists between the degree of CHD6 degradation induced by the virus and the ability of the corresponding reconstituted polymerase to degrade. The mechanism involved in the virus-induced CHD6 degradation remains uncharacterized. The fact that the sole expression of the viral polymerase is sufficient to trigger the degradation and that it occurs in the presence of a proteasome inhibitor suggests that the viral polymerase may be the main factor responsible for CHD6 degradation, although the possible role of a viral polymerase-induced cellular protease cannot be discarded. Further characterization is necessary to fully describe the mechanism involved in the degradation process.

CHD6 is degraded in mice upon influenza virus infection. The influenza virus is capable of infecting mice, so we therefore explored the possible CHD6 degradation in this animal model. With that aim, mice were intranasally mock infected or infected with 5×10^5 PFU of a recombinant influenza virus containing the RNP genes from the VIC strain and all other genes from the WSN strain [virus referred to as VIC (RNP)+WSN] (35). This reas-

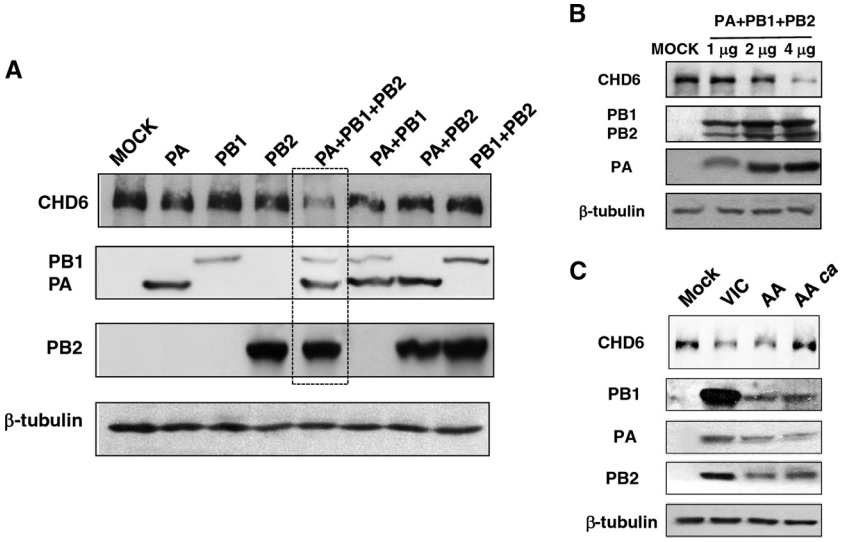

FIG 6 Reconstituted viral polymerase degrades CHD6. (A) HEK293T cells were transfected with plasmids expressing PA, PB1, or PB2 from the VIC strain individually, with combinations of two of them or with PA, PB1, and PB2 together as indicated. At 24 h posttransfection, the amount of CHD6 and the indicated proteins were detected by Western blotting. (B) HEK293T cells were left untransfected (MOCK) or transfected with increasing amounts of plasmids expressing PA, PB1, and PB2 from the VIC strain. At 24 h posttransfection, the amount of CHD6 and the indicated proteins were detected by Western blotting. (C) HEK293T cells were transfected with plasmids expressing PA, PB1, and PB2 from VIC, AA, or AA*ca* strains at 32°C. At 24 h posttransfection, the amount of CHD6 and the indicated proteins were detected by Western blotting.

sortant virus was used since we have characterized CHD6 interaction with the viral polymerase of the VIC strain (25). Furthermore, as opposed to the complete parental VIC virus, the VIC (RNP)+WSN virus is fully pathogenic in the mice model (35) and causes degradation of CHD6 in cultured cells (Fig. 7A). A total of 22 mice were used; four of them were mock infected, whereas the other 18 were influenza virus infected. At days 2, 5, and 7 postinfection, six mice were sacrificed and viral titers in the lungs were measured. The mock-infected animals were sacrificed at day 7 after inoculation. Body weight of mock- or virus-infected mice was monitored daily for 7 days with no loss observed in mock-infected mice, whereas those infected showed a 20% body weight loss 5 to 7 days after inoculation (Fig. 7B). Since the virus primarily infects the lungs of mice, lung samples of the infected animals were used to determine the viral titers that increased concomitantly with body weight loss (Fig. 7C). Since the VIC (RNP)+WSN virus contains viral genomes from A/WSN/33 and this virus is known to infect mouse extrapulmonary tissues (59),

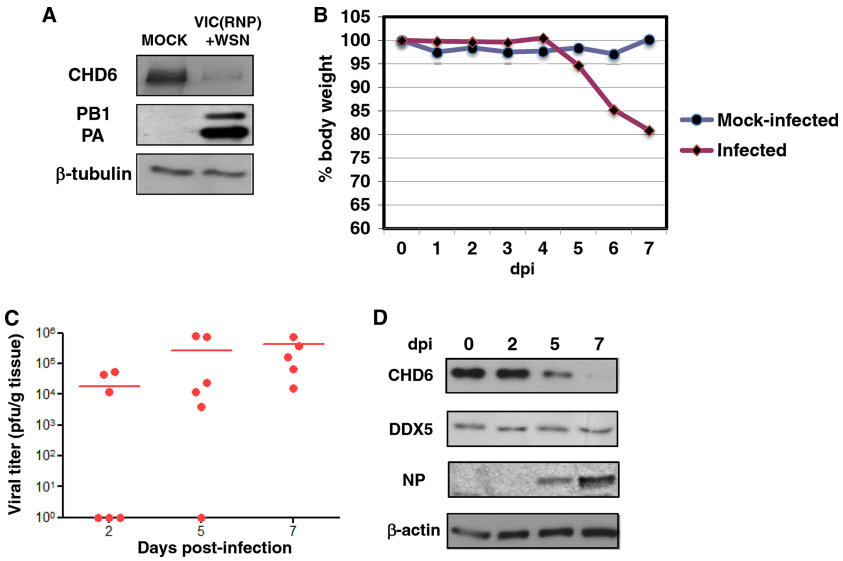

FIG 7 CHD6 is degraded in influenza virus-infected mice. (A) An influenza virus containing the RNP genes from the VIC strain and all other genes from the WSN strain was used to infect A549 cells, and the accumulation of the indicated proteins was examined by Western blotting. (B) BALB/c mice were mock infected (Control) or were inoculated intranasally with 5×10^5 PFU of the recombinant virus used in panel A. Mice body weight was evaluated daily. (C) At the indicated days postinfection, six mice were sacrificed and viral titers in the lung extracts were measured. (D) Detection of CHD6 and the indicated proteins in lungs or spleens of mock-infected and infected mice by Western blotting.

we analyzed the possible presence of viral particles in the spleen of the infected mice, but no viral titers were detected (data not shown). For protein analysis, lung homogenates from the sacrificed mice were prepared and used for Western blot assays to detect viral nucleoprotein (NP), CHD6, as well as an additional influenza virus polymerase-interacting protein that is not degraded in cell culture upon infection, DDX5 protein (14, 60). As can be seen (Fig. 7D), the accumulation of viral protein increased during the infection time, while CHD6 lung levels decreased concomitantly with the progression of the infection, being almost undetectable at day 7 concurrently with the reduction of mice body weight (Fig. 7B) and with the increase in viral titers (Fig. 7C). The six infected mice showed the same CHD6 degradation pattern, and a representative series is presented. Furthermore, no variations were found in DDX5 (Fig. 7D), another viral polymerase-interacting protein. Therefore, CHD6 would appear to be a specific target for the degradation triggered by influenza virus infection in an animal model.

DISCUSSION

Viruses exert different mechanisms in trying to overcome the defenses of the host cell in order to develop their life cycles. Frequently observed among them are mechanisms aimed at degrading the host cell components that negatively regulate the viral life cycle or are involved in the host immune response. Accordingly, DNA viruses such as adenovirus (61, 62) or Epstein-Barr virus (63) induce degradation of the transcription factor p53, which protects against virus replication. In adenovirus infection, the E1B-55k protein forms a complex with another adenoviral protein, E4-ORF6, recruiting a cellular ubiquitin ligase that targets p53 for degradation (61, 62). Other examples of DNA viruses that induce degradation-dependent viral defense strategies include degradation of the PML (64) and IRF3 (65) proteins by the bICP0 component of the bovine herpesvirus-1 virus. Furthermore, the cellular Daxx protein that represses human cytomegalovirus gene expression is degraded by the viral pp71 protein (66). In addition, epigenetic mechanisms preventing host cell transcription are also observed. Adenovirus silences p53-activated transcription by forming a nuclear structure that induces *de novo* H3K9me3 heterochromatin formation at p53 target promoters, preventing p53-DNA binding (67). RNA viruses also induce degradation processes to overcome the host defenses. Hence, the NSs protein of the Rift Valley fever virus promotes the degradation of double-stranded RNA-dependent protein kinase (68) and the basal transcription factor TFIIH (69). Furthermore, other RNA viruses induce degradation of components of the interferon cascade such as IRF3 in porcine reproductive and respiratory syndrome virus infection (70) and rotavirus infection (71), as well as STAT degradation in human respiratory syncytial virus infection (72). All this information points out that viruses commonly use protein degradation processes to allow satisfactory viral genome expression.

Proteomic analysis, two-hybrid screenings, RNA interference analyses, and functional genomic approaches have revealed an extensive network of interactions of influenza virus polymerase with the host cell (for a review, see reference 73). In agreement with the functional coupling between viral and cellular RNA metabolism, many of these factors are involved in the cellular RNA life cycle, such as the RNAP II itself (11), the cyclin T/CDK9 stimulator of transcription elongation (74), the hCLE protein, a positive modulator of the RNAP II (18, 20), the splicing factor SFPQ/PSF (14, 17), or the RNA-associated proteins DDX17 and NPM1 (75). All of the above-reported interactions, as well as the vast majority of the polymerase-interacting host factors reported, positively regulate viral replication or viral polymerase activity. In contrast, very few viral repressors have been described. In fact, from a recent genomic screening, only two factors that negatively modulate viral polymerase activity were found (75), and, furthermore, the physiological role of only a very few cases of influenza virus repressors has been characterized. Standing out among these are the characterization of Ebp-1, a suppressor of the androgen-mediated transcription that inhibits RNA synthesis of the viral polymerase *in vitro* (76); cyclophilin E, which negatively regulates virus life cycle in infected cells (77); annexin 6, which impairs virus budding (78); and the HAX1 protein, which impedes the nuclear translocation of the PA polymerase subunit (79).

The influenza virus needs to reconcile two contradictory activities. It requires an active host cell transcription to allow the cap-snatching process, but that means an active cell capable of eliciting the antiviral response. To handle these opposing requirements, impairment of host cell transcription occurs once viral transcription is finished and therefore active cellular transcription is dispensable. Accordingly, during infection, there is degradation of RNAP II with a concomitant inhibition of cellular transcription at the midpoint postinfection, an infection time at which viral transcription is already accomplished (33, 80). Moreover, inhibition of cleavage and polyadenylation of cellular pre-mRNAs (81, 82) and nuclear retention of poly(A)-containing cellular mRNAs (83) are also observed. Besides, degradation of cytoplasmic cellular mRNAs (45, 84, 85) and preferential utilization of the translation machinery by the virus-specific mRNAs (86) cooperate to efficiently shut off the expression of the host genes while maintaining an efficient expression of viral proteins. Therefore, the influenza virus triggers a battery of actions aimed at efficiently impairing the expression of genes involved in the antiviral response. CHD6 activity remains largely uncharacterized, but several reports support its function as a transcriptional activator. In this sense, CHD6 is present at intranuclear sites of mRNA synthesis (24), it operates as a coactivator for the cellular Nrf2 transcription factor (87), and it has preferential colocalization with markers of active chromatin in human epithelial lung cells (25). Interestingly, it has recently been reported that Nrf2 also acts as a negative modulator of influenza virus replication (88). Moreover, a negative role of CHD6 on human papillomavirus (HPV) gene expression has also been reported (89).

In addition to the above-described actions induced by the influenza virus to ensure a proper viral genome expression, we have described here how the viral repressor CHD6 is degraded concomitantly with RNAP II in what would seem to be yet another viral strategy to subvert and control cellular mRNA synthesis. Interestingly, all viruses that we tested of different subtypes and origins can trigger degradation of CHD6. PR8-attenuated virus was also capable of inducing this process but, as previously shown, could not degrade RNAP II. Although we still don't understand the reason for this difference, we can envision two possibilities: (i) the proteolytic actions responsible for the degradation of both proteins are unrelated, maybe through different pathways in response to different viral insults; or (ii) the same action causes the degradation of both proteins, but CHD6 is either an upstream degradation target or a more sensitive one. We favor the latter possibility since, as described in this paper and others, degrada-

tion of CHD6 and RNAP II share important characteristics, mainly not being dependent on the cellular proteasome and occurring in cells transfected with the viral polymerase. Regardless of what mechanism is responsible for CHD6 degradation during influenza virus infection, it is diminished in AA*ca*-infected cells since only a weak reduction in CHD6 protein levels is observed (Fig. 4B). The fact that we were able to recapitulate the same behavior in cells transfected with AA*ca* viral polymerase highlights the importance of this viral complex in CHD6 protein degradation. Besides this degradation, the CHD6 recruitment to histones with epigenetic marks of inactive chromatin also takes place in the infected cells (25). These actions would diminish the cellular transcription activity, thus hijacking the infected cell metabolism and favoring viral RNA replication that persists through the infection time (80) and/or reduces the antiviral response. Very importantly, CHD6 degradation occurs both in influenza virus-infected human lung epithelial cells as well as *in vivo* in the lungs of infected mice. Influenza viruses infect mainly the lung epithelium, and it should be noted that CHD6 degradation correlates with an increase in viral titers, suggesting a physiological role for this process. Therefore, the influenza virus exerts accumulative actions to inactivate the CHD6 viral repressor, causing its degradation together with its relocalization to inactive chromatin in a process that resembles p53 regulation by adenovirus. In addition, CHD6 degradation also takes place *in vivo* in the lungs of the infected mice, suggesting that CHD6 plays an important role in modulating influenza virus replication.

ACKNOWLEDGMENTS

We are indebted to J. Ortín, A. Falcón, and P. Gastaminza for their criticisms of the manuscript. We profoundly appreciate the cooperation of K. Subbarao, Y. Matsuoka, and C. Santos for providing plasmids expressing the subunits of the AA*ca* strain and AA and AA*ca* viruses. The invaluable contribution of C. Dunn for correcting the manuscript must be recognized. The technical assistance of Y. Fernández and N. Zamarreño is also gratefully acknowledged.

This work was supported by the Ministerio de Ciencia e Innovación, Plan Nacional de Investigacion Científica, Desarrollo e Innovacion Tecnologica (BFU2008-00448 and BFU2011-26175), Ciber de Enfermedades Respiratorias and Programa de investigación sobre la gripe pándemica (ISCIII). R. Alfonso was a fellow from the Ministry of Science and Education.

REFERENCES

1. **de la Luna S, Martín J, Portela A, Ortín J.** 1993. Influenza virus naked RNA can be expressed upon transfection into cells coexpressing the three subunits of the polymerase and the nucleoprotein from SV40 recombinant viruses. J. Gen. Virol. **74**:535–539.
2. **Detjen BM, St Angelo C, Katze MG, Krug RM.** 1987. The three influenza virus polymerase (P) proteins not associated with viral nucleocapsids in the infected cell are in the form of a complex. J. Virol. **61**:16–22.
3. **Horisberger MA.** 1980. The large P proteins of influenza A viruses are composed of one acidic and two basic polypeptides. Virology **107**:302–305.
4. **Huang TS, Palese P, Krystal M.** 1990. Determination of influenza virus proteins required for genome replication. J. Virol. **64**:5669–5673.
5. **Kimura N, Mishida M, Nagata K, Ishihama A, Oda K, Nakada S.** 1992. Transcription of a recombinant influenza virus RNA in cells that can express the influenza virus RNA polymerase and nucleoprotein genes. J. Gen. Virol. **73**:1321–1328.
6. **Plotch SJ, Bouloy M, Ulmanen I, Krug RM.** 1981. A unique cap(m7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. Cell **23**:847–858.
7. **Lamb RA, Lai CJ, Choppin PW.** 1981. Sequences of mRNAs derived from genome RNA segment 7 of influenza virus: colinear and interrupted mRNAs code for overlapping proteins. Proc. Natl. Acad. Sci. U. S. A. **78**:4170–4174.
8. **Shih SR, Suen PC, Chen YS, Chang SC.** 1998. A novel spliced transcript of influenza A/WSN/33 virus. Virus Genes **17**:179–183.
9. **Lamb RA, Lai C-J.** 1982. Spliced and unspliced messenger RNAs synthesized from cloned influenza virus M DNA in an SV40 vector: expression of the influenza virus membrane protein (M1). Virology **123**:237–256.
10. **Amorim MJ, Read EK, Dalton RM, Medcalf L, Digard P.** 2007. Nuclear export of influenza A virus mRNAs requires ongoing RNA polymerase II activity. Traffic **8**:1–11.
11. **Engelhardt OG, Smith M, Fodor E.** 2005. Association of the influenza A virus RNA-dependent RNA polymerase with cellular RNA polymerase II. J. Virol. **79**:5812–5818.
12. **Honda A.** 2008. Role of host protein Ebp1 in influenza virus growth: intracellular localization of Ebp1 in virus-infected and uninfected cells. J. Biotechnol. **133**:208–212.
13. **Polager S, Ginsberg D.** 2008. E2F—at the crossroads of life and death. Trends Cell Biol. **18**:528–535.
14. **Jorba N, Juarez S, Torreira E, Gastaminza P, Zamarreno N, Albar JP, Ortin J.** 2008. Analysis of the interaction of influenza virus polymerase complex with human cell factors. Proteomics **8**:2077–2088.
15. **Bates GJ, Nicol SM, Wilson BJ, Jacobs AM, Bourdon JC, Wardrop J, Gregory DJ, Lane DP, Perkins ND, Fuller-Pace FV.** 2005. The DEAD box protein p68: a novel transcriptional coactivator of the p53 tumour suppressor. EMBO J. **24**:543–553.
16. **Rosonina E, Ip JYY, Calarco JA, Bakowski MA, Emili A, McCraken S, Tucker P, Ingles CJ, Blencowe BJ.** 2005. Role for PSF in mediating transcriptional activator-dependent stimulation of pre-mRNA processing *in vivo*. Mol. Cell. Biol. **25**:6734–6746.
17. **Landeras-Bueno S, Jorba N, Perez-Cidoncha M, Ortin J.** 2011. The splicing factor proline-glutamine rich (SFPQ/PSF) is involved in influenza virus transcription. PLoS Pathog. **7**:e1002397. doi:10.1371/journal.ppat.1002397.
18. **Huarte M, Sanz-Ezquerro JJ, Roncal F, Ortín J, Nieto A.** 2001. PA subunit from influenza virus polymerase complex interacts with a cellular protein with homology to a family of transcriptional activators. J. Virol. **75**:8597–8604.
19. **Pérez-González A, Rodriguez A, Huarte M, Salanueva IJ, Nieto A.** 2006. hCLE/CGI-99, a human protein that interacts with the influenza virus polymerase, is a mRNA transcription modulator. J. Mol. Biol. **362**:887–900.
20. **Rodriguez A, Pére-González A, Nieto A.** 2011. Cellular human CLE/c14orf166 protein interacts with influenza virus polymerase and is required for virl replication. J. Virol. **85**:12062–12066.
21. **Clapier CR, Cairns BR.** 2009. The biology of chromatin remodeling complexes. Annu. Rev. Biochem. **78**:273–304.
22. **Flanagan JF, Mi LZ, Chruszcz M, Cymborowski M, Clines KL, Kim Y, Minor W, Rastinejad F, Khorasanizadeh S.** 2005. Double chromodomains cooperate to recognize the methylated histone H3 tail. Nature **438**:1181–1185.
23. **Sims RJ, III, Chen CF, Santos-Rosa H, Kouzarides T, Patel SS, Reinberg D.** 2005. Human but not yeast CHD1 binds directly and selectively to histone H3 methylated at lysine 4 via its tandem chromodomains. J. Biol. Chem. **280**:41789–41792.
24. **Lutz T, Stoger R, Nieto A.** 2006. CHD6 is a DNA-dependent ATPase and localizes at nuclear sites of mRNA synthesis. FEBS Lett. **580**:5851–5857.
25. **Alfonso R, Lutz T, Rodriguez A, Chavez JP, Rodriguez P, Gutierrez S, Nieto A.** 2011. CHD6 chromatin remodeler is a negative modulator of influenza virus replication that relocates to inactive chromatin upon infection. Cell. Microbiol. **13**:1894–1906.
26. **Bui M, Wills EG, Helenius A, Whittaker GR.** 2000. Role of the influenza virus M1 protein in nuclear export of viral ribonucleoproteins. J. Virol. **74**:1781–1786.
27. **Bukrinskaya AG, Vorkunova GK, Vorkunova NK.** 1979. Cytoplasmic and nuclear input virus RNPs in influenza virus-infected cells. J. Gen. Virol. **45**:557–567.
28. **Jackson DA, Caton AJ, McCready SJ, Cook PR.** 1982. Influenza virus RNA is synthesized at fixed sites in the nucleus. Nature **296**:366–368.
29. **Lopez-Turiso JA, Martinez C, Tanaka T, Ortin J.** 1990. The synthesis of influenza virus negative-strand RNA takes place in insoluble complexes present in the nuclear matrix fraction. Virus Res. **16**:325–337.
30. **Garcia-Robles I, Akarsu H, Muller CW, Ruigrok RW, Baudin F.** 2005.

Interaction of influenza virus proteins with nucleosomes. Virology **332**: 329–336.
31. **Takizawa N, Watanabe K, Nouno K, Kobayashi N, Nagata K.** 2006. Association of functional influenza viral proteins and RNAs with nuclear chromatin and sub-chromatin structure. Microbes Infect. **8**:823–833.
32. **Rodriguez A, Perez-Gonzalez A, Hossain MJ, Chen LM, Rolling T, Perez-Brena P, Donis R, Kochs G, Nieto A.** 2009. Attenuated strains of influenza A viruses do not induce degradation of RNA polymerase II. J. Virol. **83**:11166–11174.
33. **Rodriguez A, Pérez-Gonzalez A, Nieto A.** 2007. Influenza virus infection causes specific degradation of the largest subunit of cellular RNA polymerase II. J. Virol. **81**:5315–5324.
34. **Vreede FT, Chan AY, Sharps J, Fodor E.** 2010. Mechanisms and functional implications of the degradation of host RNA polymerase II in influenza virus infected cells. Virology **396**:125–134.
35. **Huarte M, Falcón A, Nakaya Y, Ortín J, García-Sastre A, Nieto A.** 2003. Threonine 157 of influenza virus PA polymerase subunit modulates RNA replication in infectious viruses. J. Virol. **77**:6007–6013.
36. **Falcón A, Marión R, Zürcher T, Gomez P, Portela A, Nieto A, Ortín J.** 2004. Defective RNA replication and late gene expression in temperature-sensitive influenza viruses expressing deleted forms of the NS1 protein. J. Virol. **78**:3880–3888.
37. **Sambrook J, Fritsch EF, Maniatis T.** 1989. Molecular cloning. A laboratory manual, second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
38. **Wigler M, Pellicer A, Silverstein S, Axel R, Urlaub G, Chasin L.** 1979. DNA-mediated transfer of the adenine phosphoribosyltranferase locus into mammalian cells. Proc. Natl. Acad. Sci. U. S. A. **76**:1373–1376.
39. **Bárcena J, Ochoa M, de la Luna S, Melero JA, Nieto A, Ortín J, Portela A.** 1994. Monoclonal antibodies against the influenza virus PB2 and NP polypeptides interfere with the initiation step of viral mRNA synthesis *in vitro*. J. Virol. **68**:6900–6909.
40. **Gonzalez S, Ortín J.** 1999. Distinct regions of influenza virus PB1 polymerase subunit recognize vRNA and cRNA templates. EMBO J. **18**:3767–3775.
41. **Coloma R, Valpuesta JM, Arranz R, Carrascosa JL, Ortin J, Martin-Benito J.** 2009. The structure of a biologically active influenza virus ribonucleoprotein complex. PLoS Pathog. **5**:e1000491. doi:10.1371/journal.ppat.1000491.
42. **Kawakami E, Watanabe T, Fujii K, Goto H, Watanabe S, Noda T, Kawaoka Y.** 2011. Strand-specific real-time RT-PCR for distinguishing influenza vRNA, cRNA, and mRNA. J. Virol. Methods **173**:1–6.
43. **Aragón T, de la Luna S, Novoa I, Carrasco L, Ortín J, Nieto A.** 2000. Eukaryotic translation initiation factor 4GI is a cellular target for NS1 protein, a translational activator of influenza virus. Mol. Cell. Biol. **20**: 6259–6268.
44. **Garfinkel MS, Katze MG.** 1993. Translational control by influenza virus. Selective translation is mediated by sequences within the viral mRNA 5′-untranslated region. J. Biol. Chem. **268**:22223–22226.
45. **Beloso A, Martínez C, Valcárcel J, Fernández-Santarén J, Ortín J.** 1992. Degradation of cellular mRNA during influenza virus infection: its possible role in protein synthesis shutoff. J. Gen. Virol. **73**:575–581.
46. **Katze MG, Krug RM.** 1984. Metabolism and expression of RNA polymerase II transcripts in influenza virus-infected cells. Mol. Cell. Biol. **4**:2198–2206.
47. **Beare AS, Schild GC, Craig JW.** 1975. Trials in man with live recombinants made from A/PR/8/34 (H0 N1) and wild H3 N2 influenza viruses. Lancet **ii**:729–732.
48. **Subbarao K, Chen H, Swayne D, Mingay L, Fodor E, Brownlee G, Xu X, Lu X, Katz J, Cox N, Matsuoka Y.** 2003. Evaluation of a genetically modified reassortant H5N1 influenza A virus vaccine candidate generated by plasmid-based reverse genetics. Virology **305**:192–200.
49. **Joseph T, McAuliffe J, Lu B, Vogel L, Swayne D, Jin H, Kemble G, Subbarao K.** 2008. A live attenuated cold-adapted influenza A H7N3 virus vaccine provides protection against homologous and heterologous H7 viruses in mice and ferrets. Virology **378**:123–132.
50. **Chen Z, Santos C, Aspelund A, Gillim-Ross L, Jin H, Kemble G, Subbarao K.** 2009. Evaluation of live attenuated influenza a virus h6 vaccines in mice and ferrets. J. Virol. **83**:65–72.
51. **Rieder M, Conzelmann KK.** 2009. Rhabdovirus evasion of the interferon system. J. Interferon Cytokine Res. **29**:499–509.
52. **Whelan SP, Barr JN, Wertz GW.** 2004. Transcription and replication of nonsegmented negative-strand RNA viruses. Curr. Top. Microbiol. Immunol. **283**:61–119.
53. **Yuan H, Yoza BK, Lyles DS.** 1998. Inhibition of host RNA polymerase II-dependent transcription by vesicular stomatitis virus results from inactivation of TFIID. Virology **251**:383–392.
54. **McGowan JJ, Wagner RR.** 1981. Inhibition of cellular DNA synthesis by vesicular stomatitis virus. J. Virol. **38**:356–367.
55. **Fresco LD, Kurilla MG, Keene JD.** 1987. Rapid inhibition of processing and assembly of small nuclear ribonucleoproteins after infection with vesicular stomatitis virus. Mol. Cell. Biol. **7**:1148–1155.
56. **Her LS, Lund E, Dahlberg JE.** 1997. Inhibition of Ran guanosine triphosphatase-dependent nuclear transport by the matrix protein of vesicular stomatitis virus. Science **276**:1845–1848.
57. **Zhai ZH, Nickerson JA, Krochmalnic G, Penman S.** 1987. Alterations in nuclear matrix structure after adenovirus infection. J. Virol. **61**:1007–1018.
58. **Everett RD, Lomonte P, Sternsdorf T, van Driel R, Orr A.** 1999. Cell cycle regulation of PML modification and ND10 composition. J. Cell Sci. **112**(Part 24):4581–4588.
59. **Fan K, Jia Y, Wang S, Li H, Wu D, Wang G, Chen JL.** 2012. Role of Itk signalling in the interaction between influenza A virus and T-cells. J. Gen. Virol. **93**:987–997.
60. **Jorba N.** 2009. Complejos intracelulares formados por la RNA polimerasa del virus de la gripe: oligomerización, factores asociados y complementación génica. Ph.D. dissertation. Universidad Autónoma de Madrid, Madrid, Spain.
61. **Harada JN, Shevchenko A, Pallas DC, Berk AJ.** 2002. Analysis of the adenovirus E1B-55K-anchored proteome reveals its link to ubiquitination machinery. J. Virol. **76**:9194–9206.
62. **Querido E, Blanchette P, Yan Q, Kamura T, Morrison M, Boivin D, Kaelin WG, Conaway RC, Conaway JW, Branton PE.** 2001. Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex. Genes Dev. **15**:3104–3117.
63. **Sato Y, Shirata N, Kudoh A, Iwahori S, Nakayama S, Murata T, Isomura H, Nishiyama Y, Tsurumi T.** 2009. Expression of Epstein-Barr virus BZLF1 immediate-early protein induces p53 degradation independent of MDM2, leading to repression of p53-mediated transcription. Virology **388**:204–211.
64. **Gaudreault N, Jones C.** 2011. Regulation of promyelocytic leukemia (PML) protein levels and cell morphology by bovine herpesvirus 1 infected cell protein 0 (bICP0) and mutant bICP0 proteins that do not localize to the nucleus. Virus Res. **156**:17–24.
65. **Saira K, Zhou Y, Jones C.** 2007. The infected cell protein 0 encoded by bovine herpesvirus 1 (bICP0) induces degradation of interferon response factor 3 and, consequently, inhibits beta interferon promoter activity. J. Virol. **81**:3077–3086.
66. **Hwang J, Kalejta RF.** 2007. Proteasome-dependent, ubiquitin-independent degradation of Daxx by the viral pp71 protein in human cytomegalovirus-infected cells. Virology **367**:334–338.
67. **Soria C, Estermann FE, Espantman KC, O'Shea CC.** 2010. Heterochromatin silencing of p53 target genes by a small viral protein. Nature **466**: 1076–1081.
68. **Habjan M, Pichlmair A, Elliott RM, Overby AK, Glatter T, Gstaiger M, Superti-Furga G, Unger H, Weber F.** 2009. NSs protein of rift valley fever virus induces the specific degradation of the double-stranded RNA-dependent protein kinase. J. Virol. **83**:4365–4375.
69. **Kalveram B, Lihoradova O, Ikegami T.** 2011. NSs protein of rift valley fever virus promotes posttranslational downregulation of the TFIIH subunit p62. J. Virol. **85**:6234–6243.
70. **Kim O, Sun Y, Lai FW, Song C, Yoo D.** 2010. Modulation of type I interferon induction by porcine reproductive and respiratory syndrome virus and degradation of CREB-binding protein by non-structural protein 1 in MARC-145 and HeLa cells. Virology **402**:315–326.
71. **Barro M, Patton JT.** 2005. Rotavirus nonstructural protein 1 subverts innate immune response by inducing degradation of IFN regulatory factor 3. Proc. Natl. Acad. Sci. U. S. A. **102**:4114–4119.
72. **Jie Z, Dinwiddie DL, Senft AP, Harrod KS.** 2011. Regulation of STAT signaling in mouse bone marrow derived dendritic cells by respiratory syncytial virus. Virus Res. **156**:127–133.
73. **Watanabe T, Watanabe S, Kawaoka Y.** 2010. Cellular networks involved in the influenza virus life cycle. Cell Host Microbe **7**:427–439.
74. **Zhang J, Li G, Ye X.** 2010. Cyclin T1/CDK9 interacts with influenza A

virus polymerase and facilitates its association with cellular RNA polymerase II. J. Virol. **84**:12619–12627.
75. **Bortz E, Westera L, Maamary J, Steel J, Albrecht RA, Manicassamy B, Chase G, Martinez-Sobrido L, Schwemmle M, Garcia-Sastre A.** 2011. Host- and strain-specific regulation of influenza virus polymerase activity by interacting cellular proteins. mBio **2**(4):e00151–00111. doi:10.1128/mBio.00151-11.
76. **Honda A, Okamoto T, Ishihama A.** 2007. Host factor Ebp1: selective inhibitor of influenza virus transcriptase. Genes Cells **12**:133–142.
77. **Wang Z, Liu X, Zhao Z, Xu C, Zhang K, Chen C, Sun L, Gao GF, Ye X, Liu W.** 2011. Cyclophilin E functions as a negative regulator to influenza virus replication by impairing the formation of the viral ribonucleoprotein complex. PLoS One **6**:e22625. doi:10.1371/journal.pone.0022625.
78. **Ma H, Kien F, Maniere M, Zhang Y, Lagarde N, Tse KS, Poon LL, Nal B.** 2012. Human annexin A6 interacts with influenza A virus protein M2 and negatively modulates infection. J. Virol. **86**:1789–1801.
79. **Hsu WB, Shih JL, Shih JR, Du JL, Teng SC, Huang LM, Wang WB.** 2012. Cellular protein HAX1 interacts with influenza A virus polymerase PA subunit and impedes its nuclear translocation. J. Virol. **87**:110–123.
80. **Shapiro GI, Gurney TJ, Krug RM.** 1987. Influenza virus gene expression: control mechanisms at early and late times of infection and nuclear-cytoplasmic transport of virus-specific RNAs. J. Virol. **61**:764–773.
81. **Chen Z, Li Y, Krug RM.** 1999. Influenza A virus NS1 protein targets poly(A)-binding protein II of the cellular 3′-end processing machinery. EMBO J. **18**:2273–2283.
82. **Nemeroff ME, Barabino SML, Li Y, Keller W, Krug RM.** 1998. Influenza virus NS1 protein interacts with the cellular 30 kDa subunit of CPSF and inhibits 3′ end formation of cellular pre-mRNAs. Mol. Cell **1**:991–1000.
83. **Fortes P, Beloso A, Ortín J.** 1994. Influenza virus NS1 protein inhibits pre-mRNA splicing and blocks RNA nucleocytoplasmic transport. EMBO J. **13**:704–712.
84. **Inglis SC.** 1982. Inhibition of host protein synthesis and degradation of cellular mRNAs during infection by influenza and herpes simplex virus. Mol. Cell. Biol. **2**:1644–1648.
85. **Zürcher T, Marión RM, Ortín J.** 2000. Protein synthesis shut-off induced by influenza virus infection is independent of PKR activity. J. Virol. **74**:8781–8784.
86. **Katze MG, DeCorato D, Krug RM.** 1986. Cellular mRNA translation is blocked at both initiation and elongation after infection by influenza virus or adenovirus. J. Virol. **60**:1027–1039.
87. **Nioi P, Nguyen T, Sherratt PJ, Pickett CB.** 2005. The carboxy-terminal Neh3 domain of Nrf2 is required for transcriptional activation. Mol. Cell. Biol. **25**:10895–10906.
88. **Kesic MJ, Simmons SO, Bauer R, Jaspers I.** 2011. Nrf2 expression modifies influenza A entry and replication in nasal epithelial cells. Free Radic. Biol. Med. **51**:444–453.
89. **Fertey J, Ammermann I, Winkler M, Stoeger R, Iftner T, Stubenrauch F.** 2010. Interaction of the papillomavirus E8-E2C protein with the cellular CHD6 protein contributes to transcriptional repression. J. Virol. **84**:9505–9515.