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PA Subunit from Influenza Virus Polymerase Complex Interacts with a Cellular Protein with Homology to a Family of Transcriptional Activators

MAITE HUARTE, JUAN JOSE SANZ-EZQUERRO,† FERNANDO RONCAL, JUAN ORTÍN, AND AMELIA NIETO*

Campus de Cantoblanco, Centro Nacional de Biotecnología (CSIC), 28049 Madrid, Spain

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The PA subunit of the influenza virus polymerase complex is a phosphoprotein that induces proteolytic degradation of coexpressed proteins. Point mutants with reduced proteolysis induction reconstitute viral ribonucleoproteins defective in replication but not in transcriptional activity. To look for cellular factors that could associate with PA protein, we have carried out a yeast two-hybrid screen. Using a human kidney cDNA library, we identified two different interacting clones. One of them was identified as the human homologue of a previously described cDNA clone from *Gallus gallus* called CLE. The human gene encodes a protein of 36 kDa (hCLE) and is expressed ubiquitously in all human organs tested. The interaction of PA and hCLE was also observed with purified proteins in vitro by using pull-down and pep-spot experiments. Mapping of the interaction showed that hCLE interacts with PA subunit at two regions (positions 493 to 512 and 557 to 574) in the PA protein sequence. Immunofluorescence studies showed that the hCLE protein localizes in both the nucleus and the cytosol, although with a predominantly cytosolic distribution. hCLE was found associated with active, highly purified virus ribonucleoproteins reconstituted in vivo from cloned cDNAs, suggesting that PA-hCLE interaction is functionally relevant. Searches in the databases showed that hCLE has 38% sequence homology to the central region of the yeast factor Cdc68, which modulates transcription by interaction with transactivators. Similar homologies were found with the other members of the Cdc68 homologue family of transcriptional activators, including the human FACT protein.

The genome of influenza A virus consists of a set of eight single-stranded RNA segments of negative polarity. These RNAs form ribonucleoproteins (RNPs) with four viral proteins: the nucleoprotein (NP) and the three subunits of the polymerase (PB1, PB2, and PA). These elements are required for both transcription and replication of the viral genome (10, 16, 18, 29).

The roles of the polymerase subunits have been partly outlined. The PB1 subunit contains sequence motifs typical of the viral RNA-dependent RNA polymerases (43), which have been shown to be essential for RNA synthesis (3), suggesting that this subunit is the polymerase itself. PB2 protein binds to CAP1 structures (4, 51) and is involved in the endonucleolytic cleavage of cellular mRNAs to generate the precursors used as primers for the viral transcription (6, 22). PA is a phosphoprotein in vivo and is a substrate of casein kinase II in vitro (47). This subunit induces a proteolytic process when expressed individually, affecting both coexpressed proteins and PA protein itself (46). The amino-terminal third of the molecule is sufficient to activate this proteolysis (48). Recently, we have reconstituted RNPs in vivo from cloned genes using PA point mutants deficient in proteolytic activity. These mutant RNPs are as active as the wild type in their transcription activity but have a lower capacity to support replication of model vRNA (42). These results are in agreement with the phenotype of virus temperature-sensitive mutants with mutations in the PA-encoding gene, suggesting a role for this subunit in virion RNA synthesis (23).

We have not found specific viral or cellular targets for the proteolytic process induced by PA. This fact, together with its function in replication and its role as a component of the polymerase complex of the virus, prompted us to look for specific cellular factors able to interact with PA that could play a role in the activity of this polymerase subunit.

MATERIALS AND METHODS

Biological materials. The COS-1 cell line (13), kindly provided by Y. Gluzman, was cultured as described previously (38). The vaccinia virus recombinant vTF7-3 (12) was kindly provided by B. Moss. Plasmids pGPA, pGPAΔ1–154, pGPAT157A, pGPB1, pGPB2, pGNPpolyA (derived from the polymerase genes of influenza A/Victoria/3/75 strain), and pT7NSΔCAT-RT have been described previously (29, 41, 42, 48). *Saccharomyces cerevisiae* HFtc (*MAT***a** *his3 GAL1-HIS3 GAL4-lacZ trp1 leu2*) was obtained from Clontech and used for the two-hybrid screen. Plasmids pGBT9 and pGAD424, used for interaction tests in the two-hybrid screen, as well as plasmids pVA3, pTD1, and pCL1, used as internal controls, were obtained from Clontech. Plasmid pHACDNA3, containing a hemagglutinin (HA) epitope, and the pRSET vectors were from Invitrogen. Antibodies recognizing the HA epitope were purchased from BAbCo. Construction of plasmid pHis-PAΔ1–454 has been reported previously (1). The preparation of antisera specific for PA, PB2, and NP has been previously described (2) .

Two-hybrid screen. The PA cDNA from plasmid pGPA was transferred to vector pGBT9, and the resulting plasmid (pGBTPA) was used to screen a human kidney cDNA fusion library cloned into the pGAD vector. With 2.5 mM 3-aminotriazole, the recombinant plasmid (pGBTPA) alone did not induce growth in histidine-free medium. The procedures for library amplification, yeast cell transformation, screening for growth in the absence of histidine, and measurement of β-galactosidase activity were those recommended in the Matchmaker protocol (Clontech). Rescue of positive pGAD plasmids was done by transformation into

* Corresponding author. Mailing address: Centro Nacional de Biotecnología (CSIC), Campus de Cantoblanco, 28049 Madrid, Spain. Phone: 91 5854914. Fax: 91 5854506. E-mail: anmartin@cnb.uam.es.

† Present address: University of Dundee, Dundee DD1 5EH, United Kingdom.

Escherichia coli MH4 (Leu$^-$) cells and selection in M9 plates lacking leucine. A cDNA clone corresponding to the human CLE (hCLE) sequence was obtained by PCR amplification from a HeLa cell library (Marathon-Ready cDNA; Clontech) by using as primers 5′-TACAAGGCGGCGTTCGACTGCCAAGAGC-3′ and 5′-GTCTGACCCTTTTCAACCTTCTAC-3′, using standard procedures. Sequencing was carried out in a Perkin-Elmer 373 automatic sequencer, using specific oligonucleotide primers.

Construction of mutants. To obtain recombinant pGEMThCLE, the PCR amplification product from the cDNA library was ligated to vector pGEMT (Promega). Plasmid pHis-hCLE was generated by ligation of the blunt-ended *Nco*I-*Not*I insert from pGEMThCLE to pRSETA digested with *Bgl*II and *Hin*dIII and blunt ended. The *Bam*HI-*Spe*I insert from pHis-hCLE was subcloned into plasmids pGEM3, pHACDNA-3, and pMalC digested with *Bam*HI and *Xba*I to obtain plasmids pGhCLE, pHAhCLE, and pMalhCLE, respectively. These plasmids express hCLE protein alone (pGhCLE), with an HA epitope (pHAhCLE), or as a maltose binding protein fusion (pMalhCLE).

Protein expression and purification. The His-hCLE, His-PAΔ1–464, or His-4GI157–550 protein was expressed in *E. coli* BL21DE3/pLysS cells harboring plasmid pHis-hCLE, pHis-PAΔ1–464, or pHis-eIF4GI157–550 (1), respectively. After induction for 2 h at 30°C with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), the cells were resuspended in a buffer containing 50 mM Tris-HCl, 500 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 0.1% NP-40, and 100 mM imidazole (pH 8.0) (supplemented before use with 1 mM phenylmethylsulfonyl fluoride, 1 mM tosylsulfonyl phenylalanyl chloromethyl ketone [TPCK], 1 mM *N*α-*p*-tosyl-L-lysine chloromethyl ketone [TLCK], and 10 mM 2-mercaptoethanol [2-ME]) and sonicated. After removal of cell debris by centrifugation, the supernatant was incubated with Ni^{2+}-nitrilotriacetic acid-agarose resin (Invitrogen), equilibrated in the same buffer, by rocking overnight at 4°C. After extensive washes with 20 mM Tris-HCl–0.1 M KCl–5 mM $MgCl_2$–10% glycerol–10 mM 2-ME–50 mM imidazole (pH 8.0) (washing buffer), the proteins were eluted with 1 M imidazole in washing buffer.

The Mal-hCLE protein was expressed in *E. coli* BL21DE3/pLysS cells harboring plasmid pMalhCLE. After an induction with 100 μM IPTG for 2 h at 37°C, the cells were resuspended in a buffer containing 50 mM Tris-HCl, 200 mM NaCl, 0.25% Tween 20, 10 mM EDTA, and 10 mM EGTA (pH 7.5) (supplemented before use with 1 mM phenylmethylsulfonyl fluoride, 1 mM TPCK, 1 mM TLCK, and 10 mM 2-ME) (buffer A) and sonicated. After centrifugation at 8,000 × *g* for 30 min at 4°C, the supernatant was incubated with amilose resin (New England BioLabs), equilibrated in buffer A, by rocking for 2 h at 4°C. After extensive washes with buffer A, the protein was eluted with 10 mM maltose in the same buffer.

In vitro transcription-translation. Plasmid pHAhCLE, encoding HA-hCLE, was used for in vitro transcription-translation using the Promega TNT coupled system. The gene was expressed under control of the T7 promoter, and a ^{35}S-labeled methionine-cysteine mixture (1,400 μCi/ml) was added to the cell-free protein synthesis system and incubated for 2 h at 30°C. The total cell extract was used for pull-down experiments.

Western blotting. Western blotting was done as described previously (46). The following primary antibodies were used: for His-tagged proteins, a peroxidase-labeled rabbit anti-His serum (Santa Cruz Biotechnology; 1/5,000 dilution); for HA-tagged proteins, a mouse monoclonal antibody (BAbCo; 1/3,000 dilution); and for hCLE protein, a rabbit anti-hCLE serum prepared by hyperimmunization with purified His-hCLE protein (1/300 dilution).

Immunofluorescence. Cultures of COS-1 cells were transfected with 5 μg of pHA-hCLE plasmid with a mixture of cationic liposomes (2 μl/μg of DNA) (45) in serum-free Dulbecco modified Eagle medium (DMEM). They were incubated for 6 h, washed with phosphate-buffered saline (PBS), refed with fresh DMEM containing 5% fetal calf serum, and used for analysis at 24 h posttransfection. The cells were fixed with methanol at −20°C and stored in PBS. Fixed cells were incubated with specific monoclonal antibodies against the HA epitope (1/1,000 dilution) or with polyclonal antibodies against His-hCLE (1/1,000 dilution) in PBS–0.1% bovine serum albumin for 1 h at room temperature. After being washed with PBS, the cells were stained with a 1:500 dilution of Texas red-labeled donkey anti-mouse immunoglobulin antibodies and/or a 1:500 dilution of fluorescein-labeled donkey anti-rabbit immunoglobulin antibodies in PBS–0.1% bovine serum albumin for 1 h at room temperature. Finally, the preparations were washed with PBS, mounted in Mowiol (Aldrich), and photographed in a Zeiss fluorescence microscope.

Protein interaction in vitro. For pull-down assays, in vitro-translated HA-hCLE protein was incubated with either His-PAΔ1–464 or His-4GI157–550 purified proteins immobilized in Ni^{2+}-nitrilotriacetic acid-agarose-matrix in 150 mM NaCl–10 mM Tris-HCl–1.5 mM $MgCl_2$–50 mM imidazole–0.1% NP-40 (pH 8.5). After 2 h of incubation at room temperature, the resins were washed extensively with the same buffer and the protein retained was analyzed by polyacrylamide gel electrophoresis. For pep-spot analysis, collections of 126 or 122 overlapping peptides corresponding to PAΔ1–464 or to His-hCLE proteins, respectively, were synthesized on a cellulose membranes as described previously (52). Each peptide contained 12 amino acids and had a 2-amino-acid overlap with the next. The membranes were blocked with 1% low-fat milk in PBS for 3 h at room temperature. For hCLE-PA interaction assay, the membrane containing PA peptide was incubated with purified His-hCLE or His-4GI157–550 (5 μg/ml) in PBS for 2 h at room temperature. Finally, the membranes were washed with PBS and developed by Western blotting with anti-His antibodies conjugated with peroxidase (1/10,000 dilution). For anti-His-hCLE antibody characterization, the membrane containing His-hCLE peptides was incubated with the antibody and developed as described previously (52).

RNA analysis. Oligonucleotide labeling was carried out as described elsewhere (24). Northern hybridization was performed on a human multiple-tissue Northern blot from Clontech as described previously (25).

RNP purification. Cultures of COS-1 cells were infected with vTF7-3 virus at a multiplicity of infection of 5 PFU per cell. After virus adsorption for 1 h at 37°C, the cultures were washed with DMEM and transfected with a mixture of plasmids containing (for 100-mm-diameter dishes) pGPB1 (3 μg), pGPB2 (3 μg), pGPA (0.6 μg), pGNPpolyA (12 μg), pT7NSΔCAT-RT (12 μg), and pHA-hCLE (3 μg). After incubation for 24 h at 37°C, the medium was replaced by 10 ml of DMEM containing 2% fetal bovine serum and incubated for further 24 h. The cells were collected, and the RNPs were purified by use of two successive glycerol gradients as previously described (37). Active fractions were pooled and used for further analysis.

RESULTS

Identification of hCLE as a protein interacting with influenza virus PA polymerase subunit. We carried out a two-hybrid screen in yeast with PA protein as bait. Under the conditions used, transformation of *S. cerevisiae* with plasmid pGBTPA did not stimulate growth of the cells in the absence of histidine (data not shown). Cotransformation with a human kidney cDNA fusion library constructed in plasmid pGAD led to the growth of about 3,000 independent clones after screening of 4 million colonies. Thirty-two of them were positive in the β-galactosidase assay with 2.5 mM 3-aminotriazole, and two of them were still strongly positive in the presence of 10 mM 3-aminotriazole. These two clones (PAi1 and PAi13) were confirmed as positive after isolation of the plasmids and retransformation, and they fulfilled all controls in the two-hybrid interaction protocol (data not shown). We also carried out two-hybrid analysis using pGBTPAT157A and pGBTPAΔ1–154 with clones PAi1 and PAi13. These pGBT clones express GAL4 fusion proteins with a point mutation at position 157 and an N-terminal deletion of PA protein, respectively. Both mutant proteins show a decrease in PA proteolytic induction (42, 48) and were positive in the interaction assay with PAi1 and PAi13, indicating that the first N-terminal third of PA in not required for binding to these proteins. The two clones were analyzed by restriction assay and partial sequencing. Clone PAi1 had an insert of about 3 kb pertaining to a genomic DNA human sequence present in the databases (HS620E11) that contains part of the gene for a novel helicase and a domain of a possible transcription activator. Clone PAi13 contained an insert of about 400 nucleotides with an open reading frame of 168 nucleotides. The encoded polypeptide showed high homology to the protein encoded in *Gallus gallus* CLE7 cDNA (U46756). Clones PAi1 and PAi13 had no sequence homology. In view of the homology of PAi13 and CLE7, we used oligonucleotides corresponding to the 5′ and 3′ ends of the CLE7 open reading frame to isolate the corresponding sequence from a HeLa cell cDNA library, as described in Materials and

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hCLE          1 MFRRKLTALDYHNPAGFNCKDETEFRNFIVWLEDQKIGHYKIEDR----GNLRNIHSSDW
C.elegans     1 MSRRKLRAVEYDR-DYINFDDDNEIRRLIVTVEEA---HLKCRDQNWSAQMLDEQDVAKW
                * **** *::*..   :*  *:.*:*.:** :*:    * * .*:      * : .  .*

hCLE             PKFFEKYLRDVNCPFKIQDRQEAIDWLLGLAVRLEY----GDNAEKYKDLVPDNSKTADN
C.elegans        TVELEKYFTEFGAPSGCS-RAAAIDYVLNAAVQKIYEQKGGDTELCSSRLREQAEKVLEA
                 .   ***::... *   . *  ::*::*. **:  *    **.    .    :  *  :

hCLE             ATKNAEPLINLDVNNPDFKAGVMALANLLQIQRHD-DYLVMLKAIRILVQERLTQDAVAK
C.elegans        HRDSQNPLNRLDYSSPNFAENARALCSILGISAHEPDPKVLMKAACLYIAENLGDDVIAE
                   .. :** .** ..*:*  .. **..:* *. *. *  *::**       .  :*.:*:

hCLE             ANQTKEG--LPVALDKHILGFDTG-DAVLNEAAQILRLLHIEELRELQTKINEAIVAVQA
C.elegans        ETEEVLKNKKTLNINAFPIGMQAPKNGAVHFSARLLRLICLRQLRVVSRMINETLVEIQN
                   .:       .: :: . :*:::  :..:: : *::***: :.:** :.   ***::* :*

hCLE             IIADP---KTDERLGKVGR 244
C.elegans        LTMDMSKRADLKQVQYGR 253
                 :  *    ::* :  : **
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FIG. 1. Identification of the human protein that interacts with the PA subunit of influenza virus polymerase complex. A comparison of the sequences of the human protein and its homologue from *C. elegans* is shown. The boxed sequence represents the portion of the protein that interacts with PA in the two-hybrid assay.

Methods. As a result, we obtained a PCR product of 735 nucleotides that contained a coding sequence for 244 amino acids corresponding to the protein from human origin. Later we confirmed the sequence by comparison with that reported for CGI-99 mRNA (AF151857) (20). The protein from *G. gallus* contains 239 amino acids and is identical to hCLE at 81% of its positions. Comparative proteomic studies looking for human and *Caenorhabditis elegans* conserved proteins indicated the existence in the worm of a protein homologous to the human protein (20). Figure 1 shows a comparison between the human protein (hCLE [CGI-99]) and that from *C. elegans*, as well as the coding sequence contained in the PAi13 clone.

To ascertain whether the cDNA identified in the two-hybrid screen indeed corresponds to a gene expressed in human cells, we carried out Northern analysis using premade blots generated with RNAs from a variety of human organs (Clontech) and labeled PAi13 as a probe. The results are presented in Fig. 2. A major hybridization band of around 1.2 kb was apparent in every organ assayed, with an additional minor band of around 3.5 kb also present in most of them. The estimated size of 1.2 kb agrees with the reported size of 1.1 kb for the mRNA of the human protein (20).

Characterization of the hCLE protein. To characterize the hCLE protein, we generated an antiserum by immunization of rabbits with purified hCLE protein expressed in bacteria as a recombinant with an N-terminal histidine tag (His-hCLE protein) (Fig. 3A, left panel). The antibody obtained was first assayed by Western blotting. We used purified His-hCLE protein as well as a purified maltose binding protein-hCLE fusion protein (Fig. 3A, left panel) (see Materials and Methods). The antiserum detected both proteins in this assay but detected neither the maltose binding protein alone nor an unrelated His-tagged protein (His-NS1), used as negative controls, indicating that it recognizes the hCLE protein (Fig. 3A, right panel). Furthermore, the antiserum was analyzed by pep-spot using a collection of 122 peptides covering the entire His-hCLE sequence with a 2-amino-acid shift. The retained antibodies were revealed with protein A conjugated to peroxidase, as described in Materials and Methods. As a positive control the membrane was incubated with a specific anti-His antibody bound to peroxidase. The results are presented in Fig. 3B and indicated that the antiserum specifically recognizes the hCLE protein at five different regions in the sequence and is unable to recognize the poly-His N-terminal part of the recombinant protein.

Next we wanted to determine the intracellular localization of hCLE protein. To this end, we used untransfected COS-1 cells or COS-1 cells transfected with a recombinant plasmid that expresses hCLE protein with an HA epitope at its N terminus. Untransfected COS-1 cells were analyzed with the anti-His-hCLE antiserum, while an anti-HA antibody was used for the transfected cells. The staining patterns obtained with both antibodies were essentially identical and showed that the protein was mainly localized in the cytoplasm but also was present in the cell nucleus (Fig. 4). No staining was obtained when the anti-His-hCLE antibody was preadsorbed with maltose binding protein-hCLE purified protein, whereas no change in the immunofluorescence pattern was observed when the antibody was preadsorbed with maltose binding protein (data not shown).

We also attempted to detect the hCLE protein from total cellular extracts by Western assays. We used extracts obtained from COS-1 cells or COS-1 cells infected with a vaccinia virus recombinant that expresses T7 RNA polymerase (vTF7-3) and transfected with a plasmid expressing hCLE protein under control of the T7 promoter (pGhCLE). A specific reactive band of the expected size (around 36 kDa) was detectable in the extracts of plasmid-transfected cells but not in untransfected-cell extracts (Fig. 4B), suggesting that the endogenous hCLE protein is present in small amounts.

Influenza virus PA polymerase subunit and hCLE protein interact in vitro. To further characterize the association of PA

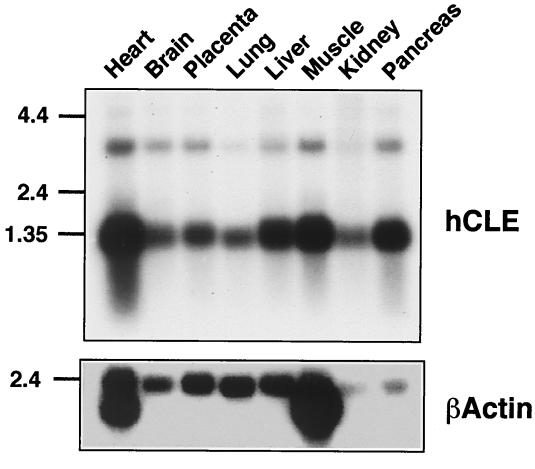

FIG. 2. Characterization of hCLE mRNA by Northern blot hybridization. Poly(A)$^+$ RNAs from human organs, separated by denaturing agarose gel electrophoresis, were probed with an hCLE-specific probe or with a β-actin probe as described in Materials and Methods. Molecular size markers (in kilobases) are indicated on the left.

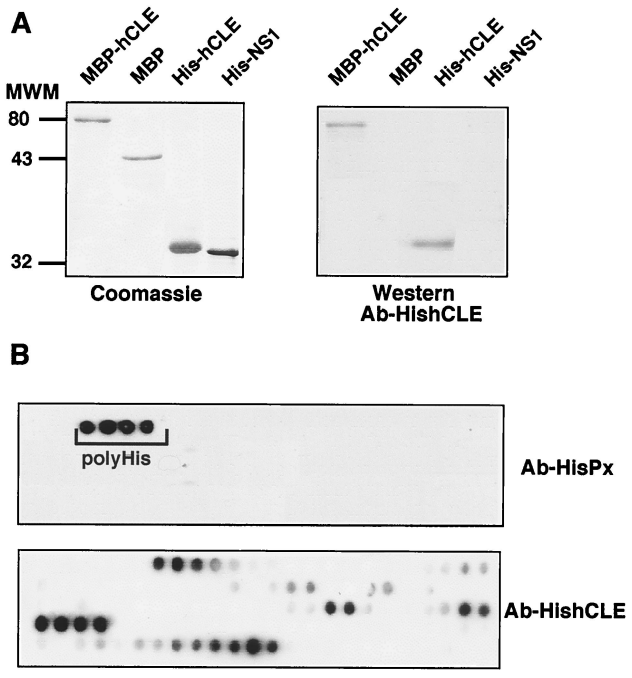

FIG. 3. Characterization of hCLE-specific antibodies (Ab). The hCLE protein was cloned as a recombinant protein with either a His or a maltose binding protein (MBP) tag at its N terminus, expressed from bacteria, and purified as described in Materials and Methods. (A) Left panel, Coomassie blue staining of the hCLE purified recombinant proteins. Right panel, Western blot obtained with the His-hCLE-specific antibody using the same preparations showed on the left. MWM, molecular weight markers (in thousands). (B) Characterization of the regions of hCLE recognized by the hCLE-specific antibody. A collection of 122 overlapping peptides representing the entire His-hCLE protein was synthesized on a cellulose membrane and used for pep-spot analysis as described in Materials and Methods. The upper panel shows the positive control reaction of the poly-His sequence with an anti-His specific antibody coupled to peroxidase (Px). The lower panel shows the different regions of hCLE recognized by the anti-His-hCLE antibody.

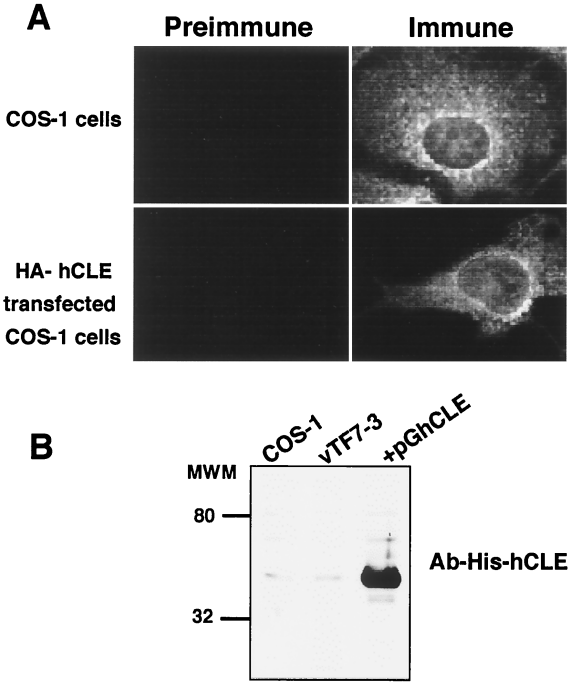

FIG. 4. Expression of hCLE protein in eukaryotic cells and subcellular localization. (A) Subcellular localization. Cultures of COS-1 cells were mock transfected (COS-1) or transfected with a plasmid expressing an HA-hCLE protein. After 24 h of incubation, the cells were fixed and processed for immunofluorescence using anti-His-hCLE (COS-1) or anti-HA (HA-hCLE transfected) specific antibodies as described in Materials and Methods. (B) Expression of hCLE in COS-1 cells. COS-1 cells were either mock transfected (COS-1), infected with vTF7-3, or infected with vTF7-3 and transfected with a plasmid expressing hCLE protein. After 24 h of incubation, total cell extracts were prepared, loaded in SDS-polyacrylamide gels, and processed for Western blotting by using His-hCLE-specific antibodies. MWM, molecular weight markers (in thousands).

and hCLE protein, we carried out in vitro binding studies. We expressed and purified from bacteria a deletion version of PA protein containing the last 252 C-terminal amino acids as a fusion protein with a histidine tag at its N terminus (His-PAΔ1–464). We used this truncated PA protein because the complete protein does not accumulate at substantial levels in bacteria and the N-terminal region is not required for interaction in the two-hybrid assay. HA-hCLE protein was labeled in vitro in a coupled transcription-translation system and incubated either with a His-PAΔ1–464-containing matrix or with a matrix bound to a histidine-tagged 4GI deletion protein (His-4GI157–550) as a negative control (Fig. 5A). After extensive washing, the retained HA-hCLE protein was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and autoradiography. The results are presented in Fig. 5B. HA-hCLE is retained in the PA-containing matrix but not in the matrix bound to the His-eIF4GI protein. The presence of the matrix-bound proteins was ascertained by Western assay with anti-His antibodies (Fig. 5B, bottom panel). These results indicate that PA and hCLE interact in vitro and localize the PA interaction domain to the last 252 amino acids of this subunit. The amount of HA-hCLE protein specifically retained by the PA-containing matrix was around 10% of the total applied protein.

To ascertain whether the hCLE-PA interaction is direct and to determine more precisely the interacting region, pep-spot analysis was carried out. A collection of 126 peptides covering the PAΔ1–464 protein with a 2-amino-acid shift was synthesized on a cellulose filter and incubated with purified His-hCLE protein or His-4GI157–550 protein as a control (Fig. 5C). The retained proteins were revealed with anti-His antibodies conjugated with peroxidase. The results are shown in Fig. 5D. A specific His-hCLE protein retention by the membrane was detected. The binding pattern revealed two regions of interaction, one between residues 493 and 512 and a second one comprising residues 557 to 574.

HA-hCLE copurifies with active RNPs reconstituted in vivo. To test the functional relevance in viral infection of hCLE-PA interaction, we asked whether this cellular protein could be present in biologically active RNPs. To that end we infected COS-1 cells with vaccinia virus vTF7-3 and transfected the cells with plasmids whose expression was T7 directed, expressing PA, PB1, PB2, and NP plus HA-hCLE proteins. The cells were also transfected with a plasmid construct that generates a

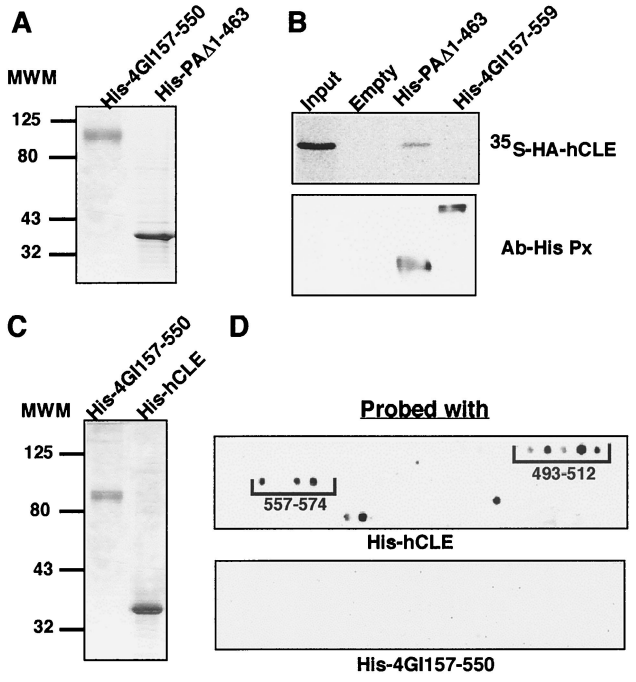

FIG. 5. PA binds in vitro-synthesized hCLE. (A and B) Pull-down assay. A plasmid expressing HA-hCLE protein was used in an in vitro transcription-translation reaction in the presence of [^{35}S]methionine-cysteine, and the synthesized protein was used for in vitro binding assays. (A) Coomassie blue staining of the recombinant proteins His-4GI157–550 and His-PAΔ1–463 that were bound to Ni^{2+} resins for the binding assay. MWM, molecular weight markers (in thousands). (B) The translation mixture was applied either to an empty Ni^{2+} matrix or to a His-PA (His-PAΔ1–463)- or His-4GI (His-4GI150–550)-containing resin. After 1 h of incubation, the resins were washed as described in Materials and Methods. In vitro translation reaction products (input) and samples of the retained protein were analyzed by SDS-polyacrylamide gel electrophoresis and exposed for autoradiography. The same samples were analyzed for the presence of proteins bound to the resins by Western blot assays with anti-His antibody (Ab) coupled to peroxidase (Px). (C and D) Mapping of the PA-interacting domain. (C) Coomassie blue staining of the purified proteins His-4GI157–550 and His-hCLE used in the assay. (D) A collection of 126 overlapping peptides representing the PA deletion mutant PAΔ1–463 was synthesized on a cellulose membrane, incubated with purified His-hCLE (upper panel) or His-4GI157–550 (lower panel), and used for pep-spot analysis as described in Materials and Methods. The signals obtained with the anti-His-specific antibody coupled to peroxidase are shown.

model vRNA transcript intracellularly (pT7NSΔCAT-RT), as previously described (37). By using this protocol it is possible to obtain reconstitution of RNPs that transcribe and replicate efficiently in vivo (41, 42) and can be purified biochemically by successive centrifugation on velocity and density glycerol gradients (26, 37). The analyses of the last step in the purification are presented in Fig. 6. The reconstituted RNPs were localized to fractions 3 to 13 in the density gradient by Western blotting using anti-PA and anti-NP antibodies (Fig. 6A). Furthermore, the in vitro transcription activity of the fractions strictly correlated with the presence of PA (Fig. 6A). In a control reconstitution in which the model vRNA was omitted, NP protein was also present in similar positions on the gradient, as a consequence of its ability to associate with RNA unspecifically to form aggregates (Fig. 6B). In contrast, PA protein was detected at the top of the gradient (fractions 1 to 7), and no transcription activity was detectable (Fig. 6B). The presence of the transfected HA-hCLE in the gradients was evaluated by Western blotting, and the results are presented in Fig. 6. As can be seen, the transfected protein was distributed in fractions 4 to 13, strictly correlating with reconstituted active RNPs (Fig. 6A). In contrast, HA-hCLE was present in fractions 1 to 7 in the control gradient, the same fractions where PA is localized. These results indicate that HA-hCLE is associated with active RNPs in vivo and suggest that hCLE-PA interaction is biologically relevant.

DISCUSSION

In their interaction with susceptible cells, many RNA viruses take over the cellular gene expression machinery, leading to the preferential synthesis of viral products and the shutoff of cellular expression. In addition, there are many examples of viruses that divert cellular proteins or RNAs as cofactors for their own transcription and/or replication (21). In some cases, cellular proteins participate in viral RNP complexes by binding to viral RNA, as in poliovirus, Sindbis virus, hepatitis C virus, vesicular stomatitis virus, human parainfluenza virus-3, and human immunodeficiency virus (5, 9, 15, 17, 19, 40, 54). In other examples, the cellular factor associates with the virus polymerase, as has been described for brome mosaic virus, tobacco mosaic virus, vesicular stomatitis virus, measles virus, and poliovirus (8, 28, 31, 39, 44). Many of these factors are normal components of the cellular RNA processing or translation machinery (21).

Relevance of hCLE-PA subunit interaction. In the case of influenza virus, it has been shown that cellular factors are involved in the modulation of the influenza virus RNA synthesis (32, 49), suggesting a physical and/or functional interaction between cellular proteins and influenza virus RNPs. A number of cellular proteins have been isolated as influenza virus NP-interacting factors (30, 34). Some of them belong to the importin-β family (NPI-1 and NPI-3), while another (NPI-5) is a subunit of splicing factor UAP56. Interaction with NPI-1 could mediate nuclear import of RNPs (35), and NPI-5 is a cofactor for vRNA replication in vitro that could enhance binding of NP to the nascent RNA chain (30). In this paper we describe the cloning of a human cellular protein (hCLE [CGI-99]) based on its ability to interact with the PA subunit of influenza A virus polymerase. hCLE interacts with PA subunit in the yeast two-hybrid assay and in vitro as shown by using pull-down and pep-spot experiments with purified proteins (Fig. 1 and 5). The in vitro interaction data indicate that the hCLE interaction domain maps in regions of PA protein included in its C-terminal third, close to the region of PA that interacts with the PB1 subunit of the polymerase complex (14, 27). Correspondingly, a PA mutant with a deletion of 154 amino acids at its N terminus possesses hCLE binding capacity in the two-hybrid system. This PA mutant, as well as mutant PA-T157A, is defective in proteolysis induction (42, 48) and is positive for hCLE interaction in the two-hybrid assay, indicating that this PA activity is not necessary for PA-hCLE interaction. In order to ascertain the relevance of PA-hCLE interaction for influenza virus RNA synthesis, we looked for the presence of hCLE in active RNPs reconstituted in vivo from cloned genes. Inter-

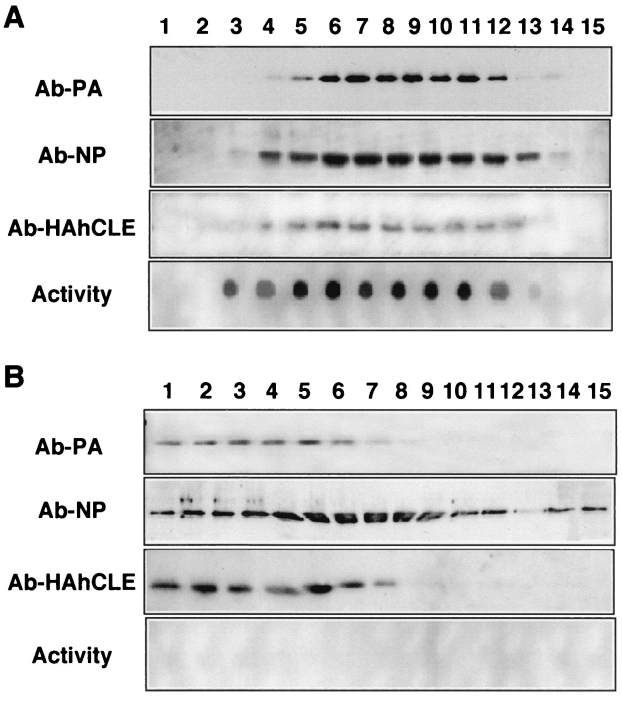

FIG. 6. HA-hCLE is present in active purified influenza virus RNPs reconstituted in vivo. Viral RNPs reconstituted in vivo in the presence or absence of a vRNA-like model were purified by use of two successive glycerol gradients as described in Material and Methods. The analyses corresponding to the fractionation of the second gradient are presented; lane numbers correspond to fraction numbers. Aliquots of each fraction were processed for Western blotting by using anti-PA, anti-NP, or anti-HA antibodies (Ab). The activity of each fraction was determined by in vitro transcription, trichloroacetic acid precipitation, filtration on a dot blot apparatus, and autoradiography. (A) RNPs reconstituted with a vRNA-like model. (B) RNPs reconstituted without a vRNA-like model.

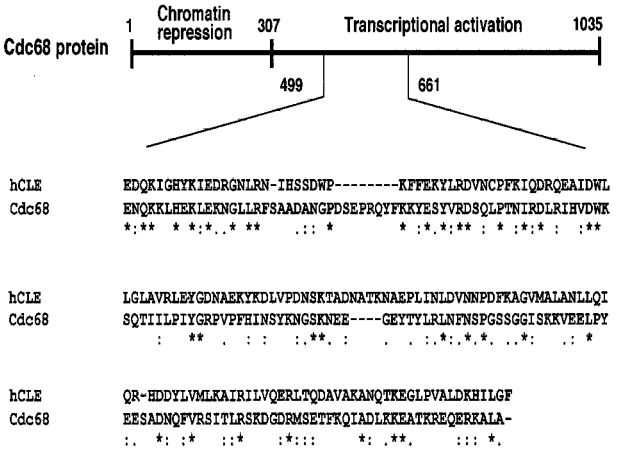

FIG. 7. Homology between hCLE and Cdc68 from the yeast *S. cerevisiae*. Upper part, Cdc68 schematic, showing the N terminus and the region required for transcriptional activation. Lower part, comparison of the hCLE protein and the central part of the yeast Cdc68 protein.

estingly, coexpressed hCLE associated with extensively purified RNPs but was excluded from the corresponding fractions of a control RNP preparation in which the vRNA template was omitted. The reconstituted RNPs were transcriptionally active and pure enough to allow three-dimensional reconstruction of influenza virus RNP particles (26, 37). These results indicate that hCLE interacts not only with isolated PA protein but also with PA protein when it is forming the polymerase complex, and they suggest that hCLE-PA interaction is relevant for influenza virus replication.

What role may hCLE (CGI-99) play in influenza virus replication? Previous biological information about the hCLE gene was negligible. The gene was isolated first from *G. gallus* and later from *C. elegans* (20), but no clue about its function was reported. During this work we have carried out a preliminary characterization of the hCLE gene and protein, including comparative studies, in order to obtain hints about its possible role in influenza virus infection. By Northern analysis we have found that hCLE is expressed in all organs from human origin analyzed (Fig. 2). By immunofluorescence studies we have found that hCLE is expressed in cell lines from human (HeLa), canine (MDCK), and monkey (COS-1) origin (data not shown), suggesting a conserved role for this protein. Database searching revealed that the hCLE (CGI-99) protein is 38% homologous to the central region of the *S. cerevisiae* Cdc68 (Spt16) protein (Fig. 7). Cdc68 is a nuclear protein of 1,053 amino acids that is necessary for the transactivation of many genes as well as for the maintenance of chromatin-mediated repression in the absence of transactivators. Cdc68 associates with another yeast protein called Pob3, forming the CP complex (7). By deleting the N-terminal domain of Cdc68 it has been shown that this part of the protein is necessary for chromatin-mediated repression, and the truncated molecule is still functional as transcriptional activator (11). Proteins from several species have been found to show sequence homology to Cdc68: (i) a DNA-unwinding factor (DUF) from *Xenopus laevis* involved in DNA replication (33), (ii) a protein named Dre4 from *Drosophila melanogaster* that is developmentally regulated (50), and (iii) a human protein named FACT (for facilitated chromatin transcription) (36). This human factor is a 140-kDa protein that interacts with histone H2A-H2B dimers and promotes nucleosome disassembly upon transcription (36). Recently it has been shown that FACT performs its activity in conjunction with the RNA polymerase II CTD kinase P-TEFb and regulates transcription on naked DNA independently of its activity on chromatin templates (53). Therefore, both Cdc68 and FACT form protein complexes that function as chromatin-remodeling factors and also regulate transcription independently of their activities in nucleosome disassembly. Interestingly, hCLE has sequence homology to Cdc68 (residues 499 to 661) in the part of the protein involved in transcriptional regulation that is independent of chromatin remodeling (Fig. 7). Similar homologies appear in comparisons of hCLE and the other members of the Cdc68 homologue family of proteins such as DUF, Dre4, and FACT, suggesting that hCLE and the Cdc68 homologue family share a function exerted by this region.

In view of the common features of Cdc68, DUF, Dre, and FACT as components of transcriptional regulator multicomplexes, it is tempting to speculate that influenza virus could form RNP complexes in a fashion similar to that for DNA-dependent RNA transcription complexes. Thus, the influenza

virus polymerase might serve as a basal landing complex to assemble factors from the infected cell that may modulate the synthesis of the different types of viral RNAs. Due to the suggested role of PA in virion RNA synthesis, its interaction with hCLE could be at the basis of the mechanism by which this subunit regulates the replication activity of the polymerase.

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