J. Biochem. **136**, 169–176 (2004)
DOI: 10.1093/jb/mvh108

HTRP—An Immediate-Early Gene Product Induced by HSV1 Infection in Human Embryo Fibroblasts, Is Involved in Cellular Co-Repressors

Jian-Feng Li*, Long-Ding Liu*, Shao-Hui Ma, Yan-Chun Che, Li-Chun Wang, Cheng-Hong Dong, Hong-Ling Zhao, Yun Liao and Qi-Han Li†

Institute of Medical Biology, Chinese Academy of Medical Sciences, Peking Union Medical College, 379 Jiaoling Rd., Kunming, 650118, PR China

Received March 1, 2004; accepted May 11, 2004

The interaction between virus and receptor is a process that mimics physiological ligand binding receptors and induces signal transduction. In the investigation of the interaction between HSV1 (Herpes Simplex virus 1) and human fibroblasts via virus binding to its receptor complex on cellular membranes, the HTRP (human transcription regulator protein), a protein encoded by an immediate-early gene of cellular response against the specific stimulation of HSV1 binding, was cloned from a cDNA library established from early gene response mRNA. The localization of HTRP expressed as a fusion polypeptide with a fluorescent protein in HeLa cells was confirmed to be the nucleus. The results of a yeast two-hybrid experiment indicated that HTRP is indeed involved in the interaction with the SAP (mSin3-associate polypeptide) complex via SAP30. A pull-down test and Western blotting *in vitro*, and immunoprecipitation *in vivo* also provided evidence in support of this result. The interaction of HTRP with SAP30 in its conserved domain implies that this protein family, as the products of immediate-early genes, comprise functional molecules involved in the transcriptional regulation of cells, which might be related to the inhibition of some cell survival genes.

Key words: co-repressor, HDAC, HSV1, HTRP, SAP30, transcription regulation.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EGFP, enhanced fluorescent protein; FBS, fetal bovine serum; GST, gluthathione S-transferase; HDAC, histone deacetylase; HSV1, Herpes Simplex virus 1; HTRP, human transcription regulator protein; N-CoR, nuclear receptor co-repressor; ONPG, *o*-nitrophenyl β-D-galactopyranoside; PMSF, phenylmethylsulfonyluoride; SAP30, mSin3-associate polypeptide 30 kDa; SMRT, silencing mediator of retinoic and thyroid hormone receptors.

The interaction between virus and receptor in the cellular membrane mimics physiological ligand binding to a specific receptor, and also activates a specific signal transduction in cells (*1*). In fact, such signal activation could induce a related gene response that would likely induce an immediate-early gene response and a second gene response (*2–4*). Previous studies have indicated that the virus binding receptor induces various gene activations (*5–7*). Herpes Simplex virus 1 (HSV1), as a member of the Herpesviridae family of viruses, usually initiates its infection in cells by interaction with receptors via heparan sulfate moieties and an unidentified molecular attachment (*8*, *9*). This interaction between HSV1 and receptors has also been confirmed to induce some genes to become activated and expressed in cells infected by HSV1 (*10–13*). These genes that are activated and expressed in cells are related to the regulation of cellular transcription and the cell cycle process (*14*, *15*), and some identified functional genes and novel genes have been isolated from s cDNA library of human embryo fibroblasts infected with HSV1, as demonstrated by our previous work (*16–18*). The identification of related gene products implies that HSV1 binding to the receptor in the membrane of human fibroblasts may regulate the transcriptional level of the cellular genome via some oncogene products and novel protein molecules (*14*, *15*, *18*). One even exhibits the ability to interfere with the pre-mRNA splicing process of HSV1 in a specific cycle phase (*14*). Another shows an interesting characteristic a novel protein family, the SAND family, that tends to be related to the transcriptional regulation complex in cells (*18*). Further investigations of novel protein molecules encoded by genes activated by HSV1 binding to the receptor has led to the discovery of a protein molecule, the human transcription regulator protein (HTRP), this is involved in the cellular co-repressor complex of gene transcription, and induces the death of cells transfected by its gene. As is known, co-repressor complexes are regulators recruited by DNA-bound transcriptional silencers that play essential roles in many pathways, including differentiation, proliferation, programmed cell death and the cell cycle (*19–21*). Until now, a large number of co-repressors in eukaryotic cells have been identified as interacting with related transcription factors and mediating gene silencing. In this interaction and silencing, the activities of his-

*These authors contributed equally to this work.
†To whom correspondence should be addressed. Tel: +86-871-8335905, Fax: +86-871-8334483, E-mail address: qihanli@public.km.yn.cn

tone deacetylase (HDAC) enzymes are involved in co-repressor functions (*22*, *23*) and regulated by interactions with the partners of targeting complexes, *e.g.*, the mSin3-protein/ SAP-protein complex, nuclear receptor co-repressor (N-CoR) complex and the silencing mediator of the retinoic and thyroid hormone receptors (SMRT) complex (*20*, *24–26*). The mSin3-protein comprises mSin3A and mSin3B, which bind directly to some silencers and target their activities to these repressors in association with the SAP complex (*27–29*). Both N-CoR and the SMRT complex are important targets of the cell signaling pathway, and are able to associate with the HDAC activity providing critical components for the transcriptional repression of specific genes. Histone deacetylation 06 lysine residues in the N-terminal is known to be correlated activation and repression of genes and is determined by HDAC activity (*30*, *31*). All these results suggest a mechanism of gene regulation through the targeting enzyme that alters chromatin structure. In this case, the interaction of many components in the targeting complex should be of importance in the transcriptional regulation of cells. Here, we report that HTRP, as a protein encoded by an immediate-early gene induced by the HSV1 binding cellular receptor, was found to interact with SAP30 associated with the mSin3-complex, and to induce the death of cells transfected with its gene. The results suggest that HTRP is involved in the transcriptional regulation of cells, which is probably related to the termination of the cellular life cycle by an unknown mechanism. To obtain further understanding of this interaction between HTRP and SAP30, we focus on the mechanism by which HTRP interacts with SAP30 associated with the human histone deacetylase complex in cells.

MATERIALS AND METHODS

Cell Culture and Virus Infection—KMB-17 human embryo fibroblasts (Institute of Medical Biology, Chinese Academy of Medical Sciences, Kuming, PR China) were maintained in DMEM (Dulbecco's modified Eagle's medium, GIBCO) supplemented with 2 mM glutamine (Sigma) and 10% (v/v) fetal bovine serum (FBS, GIBCO) in an atmosphere of 5% CO_2 at 37°C. HSV1 (Institute of Virology, Beijing) ($lg10^{7.5}$CCID50/ml) was proliferated in Hep-2 cells (ATCC) and used to inoculate KMB-17 cells grown as a monolayer.

Identification and cDNA Cloning of the htrp Gene—A cDNA library was cloned from the KMB-17 cells inoculated with HSV1 for 2 h. The cDNA for the htrp gene was isolated by mRNA differential display and confirmed by sequencing. The sequence was identified in GenBank and localized in the chromosome with the Human Genome Project Working Draft website (http://genome.ucsc.edu). The secondary structure of the protein was predicted by The Predict Protein Server (http://maple.bioc.columbia.edu/predictprotein), and localized in its sub-cellular site by the PSORTII server (http://psort.nibb.ac.jp). The protein subsequence motifs were identified using the network service SMART (http://hsmart.embl-heidelberg.de). The protein domain homology was compared in Prodom (http://prodes.toulouse.inra.fr/prodom).

Plasmids Construction—Plasmid pGEX-HTRP was constructed by inserting the HTRP cDNA into the *Eco*RI sites of pGEX-5x-1 (Amersham Pharmacia Biotech) to express a GST fusion protein. Plasmid pEGFP-HTRP was constructed by inserting HTRP cDNA into the *Eco*RI sites of pEGFP-N1 (Clontech) to express a green fluorescent protein (EGFP) fusion protein. Plasmid pGBK-HTRP expressing Gal4-HTRP, was constructed by inserting the full-length HTRP cDNA into the *Eco*RI site of pGBKT7 (Clontech) plasmid. The plasmids pGBK-mtA, pGBK-mtB, pGBK-mtC, pGBK-mtAB, pGBK-mtBC and pGBK-mtD were cloned by inserting the DNA fragments encoding the corresponding peptides from HTRP, namely residues1–91, 92–225, 226–308, 1–225, 92–308, and 1–141, respectively, between the *Eco*RI and the *Xho*I sites of pGBKT7. Plasmid pcDNA-HTRP was constructed by inserting the HTRP cDNA into the *Eco*RI sites of the mammalian expression vector, pcDNA3 (Invitrogen). All plasmid DNA was confirmed by sequence analysis.

Northern Blot Analysis—Total mRNA from KMB-17 human embryo fibroblasts pre-induced and induced by HSV1, and from human multiple tissues, was used for Northern blot hybridization by the probes specific for htrp and β-actin, respectively. The probes were generated by the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP in the Prime-a-Gene® labeling system (Promega). The Northern blots were pre-made with Poly (A) + RNA from 6 human tissues (Promega). The DNA template for the htrp probe (a 500 bp fragment, nucleotides 121–621 of htrp) was a fragment that was not homologous to other sequences. The hybridization procedure followed the manufacturer's protocol. The blots was washed and exposed to X-ray film at –70°C with an intensifying screen.

Intracellular Localization of HTRP—HeLa cells plated in 6-well plates at 90% confluence in DMEM supplemented with 10% FBS were transiently transfected with control plasmid pEGFP-N1 and expression plasmids pEGFP-HTRP using Lipofectamine Plus reagent (Invitrogen). All cell samples were viewed under a fluorescence microscope 36 h after transfection .

Yeast Two-Hybrid Screen and β-Galactosidase Assay—A full-length cDNA of HTRP was cloned into pGBKT7 as a Gal4-DNA–binding domain fusion. This construction was used to screen a pretransformed human Liver cDNA library (BD Biosciences Clontech). Approximately 10^4 transformants were screened according to the manufacturer's protocol. Colonies were replated on minimal medium without leucine, tryptophan, histidine and adenine. pACT2 plasmid DNA was isolated from the yeast clones, amplified in *E. coli* and retransfected into AH 109 cells together with pGBKT7-HTRP or a negative control, pGBKT7-LaminC (Clontech).Clones expressing proteins and interacting specially with HTRP were isolated and sequenced. Analysis of the intrinsic regions of HTRP interacting with positive protein was done by the liquid β-galactosidase assay according to manufacturer's protocol (Yeast Handbook, BD Biosciences Clontech). In brief, 6 HTRP mutants were constructed and reacted with their interacting proteins in a yeast two-hybrid system. Using *o*-nitrophenyl β-D-galactopyranoside (ONPG, Sigma) as a substrate, a liquid assay was used to compare the relative strengths of protein-protein interactions observed in selected transformants. Transactivation activity was revealed in different β-galactosidase

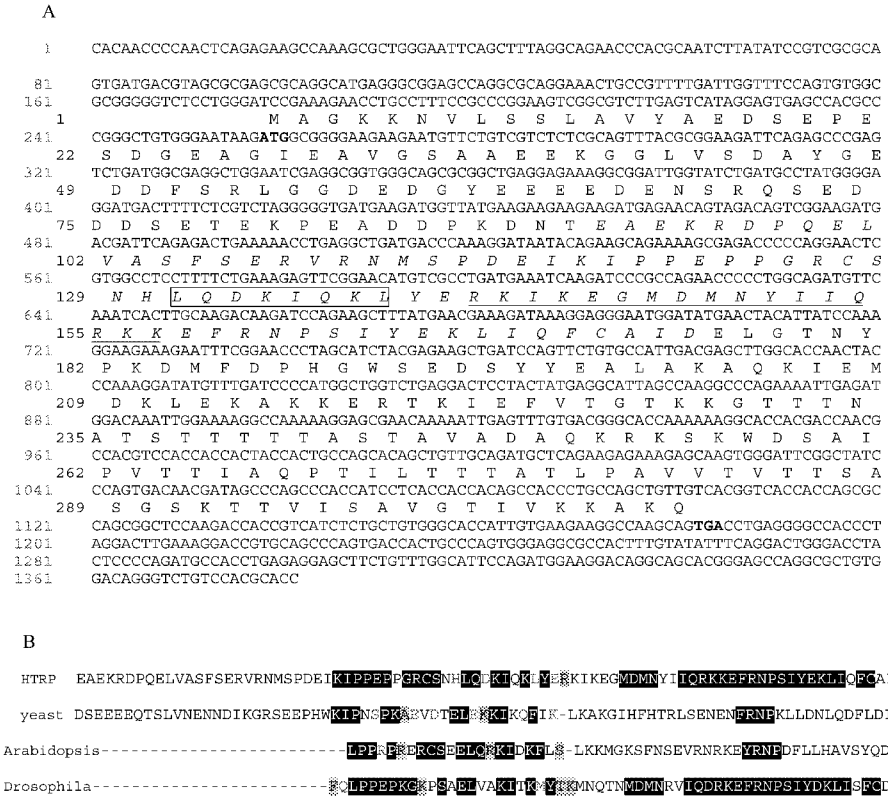

Fig. 1. **Identification and characterization of htrp cDNA and its deduced amino acid sequence.** A: htrp cDNA and its deduced amino acid sequence. Amino acids are shown by their one letter code above the corresponding nucleotide sequence. An in frame 5′ start codon and the termination stop codon are in bold letters. A bipartite nuclear location signal at residues 141–157 is underlined, and a motif at residues 131–138 similar to N-CoR proteins is boxed. A conserved domain at residues 92–175 is in italic letters. B: Conserved domain homologue comparison analysis. The conserved domain (residues 92–175) of HTRP and its homologue region from proteins in Drosophila, Arabidopsis thaliana and Fission yeast (Swiss-prot accession numbers Q9V563, Q9LP63 and Q10069, respectively) was analyzed by Vector NTI suite. The homologue region is shown in shadow.

units calculated based on optical absorption value at 420 nm.

Cytometry of Human Fibroblasts Ttransfected with the htrp Gene—KMB-17 cells plated in 6-well plates at 90–95% confluence in DMEM supplemented with 10% FBS, were transiently transfected with control plasmid pcDNA3 and expression plasmid pcDNA-HTRP with Lipofectamine Plus reagent (Invitrogen). At 12 h, 24 h, 36 h, 48 h, 60 h and 72 h post-transfection, cells were trypsinized, washed in PBS, and fixed in 70% ethanol at 4°C for 30 min. Apoptotic nuclei were detected by the TUNEL-labeling reaction according to the manufacturer's instructions (Roche Molecular Biochemicals). The cells were then washed and analyzed by flow cytometry. Each experiment was repeated at least three times.

GST Fusion Proteins Pull-Down Assay—GST and GST-HTRP were expressed in *Escherichia coli* BL21 and purified according to standard protocols. The GST and the GST-HTRP fusion proteins were incubated overnight at 4°C with a KMB-17 cell extract pre-labeled with [^{35}S]methionine (prepared in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 0.5% Na deoxycholate, and 0.1% SDS) containing phenylmethylsulfonyluoride (PMSF, Sigma), and conjugated further with glutathione-Sepharose 4B beads in a total volume of 500 μl of incubation buffer (20 mM Tris, pH 7.5, 75 mM KCl, 50 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, and PMSF. After centrifugation, the beads were washed five times with incubation buffer and resuspended in PBS buffer, boiled for 5 min, and centrifuged. The supernatant was subjected to electrophoresis in 12% SDS-polyacrylamide gels. After drying, the gels were subjected to autoradiography.

Immunoprecipitation of HTRP in Human Fibroblast—Immunoprecipitation of the HTRP protein was performed according to the standard protocol. KMB-17 cells were grown in DMEM with 5% of FBS to 80–90% confluence at 37°C, and then incubated in methionine-free MEM at 37°C for 1 h. Labeling was performed with the same media containing [^{35}S]methionine at 37°C for 2 h. One hour after virus inoculation, the cells were rinsed twice with PBS and scraped in 100 μl of RIPA buffer (150 mmol/liter NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mmol/liter Tris-HCl pH 7.5), and freeze-thawed 3 times. After centrifugation at 12,500 rpm for 10 min at 4°C, the supernatant was incubated with an anti-HTRP polyclonal antibody described previously (*16*) or with an anti-SAP30 polyclonal antibody purchased from Upstate Biotechnology in RIPA buffer at 37°C for 1 h; the A protein-Sepharose 4B (Sigma) was added for further incubation at 4°C for 1 h. After washing 3 times with RIPA buffer and centrifugation as above, the A protein–Sepharose 4B absorbed immune complex pullet was incubated in SDS sample buffer (2% SDS, 62.5 mmol/liter Tris, 10% glycerol, 2% 2-mercaptoethanol pH 6.8) at 100°C for 5 min. The supernatant was subjected to SDS-PAGE followed by centrifugation as above. Finally, the gel was dried and exposed to X-film.

Western Blotting Analysis—Proteins extracted from KMB-17 cells by GST-HTRP or the anti-HTRP antibody were fractionated in 10% polyacrylamide gels and transferred onto nitrocellulose membranes according to standard methods. The primary antibody for Western blotting was the anti-SAP30 antibody at 1 μg/ml dilution. Signals

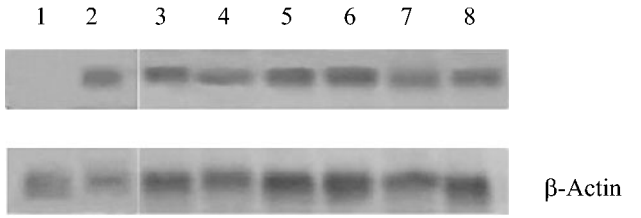

Fig. 2. **Northern blotting of htrp expression in human embryo fibroblasts bound to HSV1 and 6 tissues.** KMB-17 cells were mock infected or infected with HSV1 (M.O.I. = 1), and total mRNA was isolated for Northern blotting. The pre-transferred PVDF membranes of poly(A) + RNA of 6 human tissues were used to detect the HTRP transcript in human tissues. The HTRP-specific transcript was detected by using a probe for htrp (a fragment of 500 nt, nucleotides 121–621 of htrp). The β-actin mRNA was detected as a control by a 491 nt probe. Lane 1: human embryo fibroblast control; Lane 2: human embryo fibroblast bound to HSV1 for 2 h; Lane 3: brain; Lane 4: heart; Lane 5: liver; Lane 6: lung; Lane 7: spleen; Lane 8: kidney.

were detected by the ECL system (Pierce) according to the manufacturer's instructions.

RESULTS

Cloning of htrp cDNA and Its Deduced Amino Acid Sequence—Gene activation by a virus binding receptor is, in fact, a physiological response similar to that caused by ligand binding. This enables viruses to influence the cellular physiological processes and to establish a new cellular microenvironment, in which the components of the virus will likely be affected by the protein molecules induced in this process. Upon the analysis of the gene response induced by HSV1 binding to human fibroblasts, a cDNA gene, named htrp, was cloned from the cDNA library estabilished from the cells bound by HSV1 for 2 h (Fig. 1A) (GenBank accession number AF450482) (*16*). As a protein encoded by an immediate-early gene, HTRP was hypothesized to be involved in the transcriptional regulation of cells infected by the HSV1. In its deduced amino acid sequence, a bipartite nuclear signal—RKIKEGMDMNYIIQRKK is localized in residues 141–157, and a motif—L-X-X-X-I-X-X-L similar to that in the nuclear receptor co-repressor (N-CoR) protein comprises residues 131–138, indicating a possible function of this protein in the nucleus. Further structural comparison suggested that this protein has a homologue of a conserved domain in Drosophila, Arabidopsis thaliana and Fission yeast (Fig. 1B), but no data about the function of this homologue has been reported up to now.

Distribution of htrp Gene in Tissues—Previous studies have indicated that the role of immediate-early gene response is to regulate the expression of functional genes in cells receiving a stimulation signal (*5*, *6*, *8–12*). Our previous data suggest that the binding of HSV1 to human fibroblasts induces a remarkable change in the expression of some transcriptional regulators, such as 0N-myc, C-fos *etc*. (*14*, *15*). To obtain an understanding of the physiological expression of htrp in tissues and the relationship between its expression and HSV1 binding, northern blotting was performed. The results show that the enhanced expression of htrp with a size of 1,350 nt is

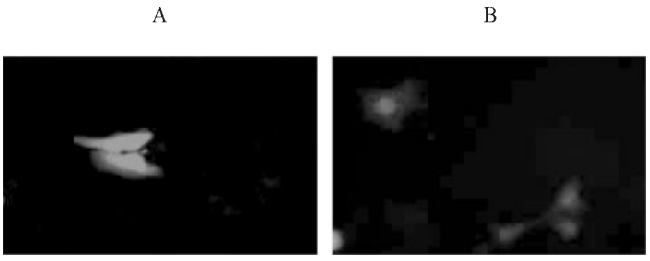

Fig. 3. **Subcellular localization of HTRP in HeLa cells.** The subcellular localization of GFP–HTRP was analyzed in transfected HeLa cells in the presence of control vector. Fluorescence was observed in live cells at 36 h after transfection (magnification, ×400). A: HeLa cells were transfected with pEGFP-N1 control plasmid, the EGFP protein was expressed and distributed in the nucleus and plasma. B: HeLa cells were transfected with pEGFP-HTRP, the HTRP-EGFP fusion protein was mainly distributed in the nucleus.

specific to human embryo fibroblasts bound to HSV1 and nonspecific in some tissues (Fig. 2), implying the involvement of htrp expression in an unknown physiological function in these tissues.

Localization of HTRP from HeLa Cells—In the structural analysis of HTRP, a bipartite nuclear localization signal was noticed, and an HTRP involved in transcriptional regulation was hypothesized. To confirm this hypothesis, the localization of HTRP was investigated. The fluorescent vector carrying the htrp gene was transfected into HeLa cells for the dynamic observation of HTRP. The results indicated that HTRP expressed in HeLa cells localizes in the nucleus (Fig. 3), which indicates that it functions in nucleus.

Expression of HTRP Induces Human Fibroblasts Death—Although the physiological function of HTRP in fibroblasts has not been investigated, some phenotypic changes of fibroblasts transfected with the htrp gene

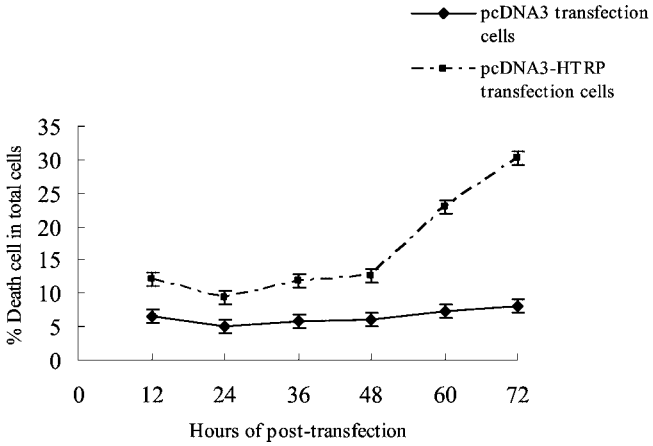

Fig. 4. **Quantitative analysis of HTRP-induced cell death.** KMB-17 cells were transfected with pcDNA3-HTRP plasmid or the control plasmid pcDNA3. At different times after transfection, the cells were labeled by TUNEL. Flow cytometry was performed on an EPIC-XL (Coulter). Wincycle 2.0 software was used to determine the percentage of death cells. The mean percentage and standard deviation of triplicate samples are shown.

Table 1. **HTRP interactions with related proteins in a yeast two-hybrid system.**[a]

DNA binding hybrid	Activation hybrid	Colony color
pGBKT7 (no insert)	pACT2	no colony
pGBK-HTRP	pACT-X1	blue
pGBK-HTRP	pACT-X2	blue
pGBK-HTRP	pACT-X3	blue
pGBK-HTRP	pACT-SAP30	blue
pGBK-HTRP	pACT2 (no insert)	no colony
pGBKT7-LaminC	pACT-SAP30	no colony
pGBKT7-p53	pGADT7-T	blue

[a]Plasmids in column 1 and column 2 were co-transfected into yeast strain AH109 as pairs. Diploids were assayed by selecting for growth in the absence of leucine, tryptophan, histidine, and adenine, and for expression of the LacZ promoter by β-gal activity according to the manufacturer's protocol (Clontech). Plasmids pACT-X1, pACT-X2, pACT-X3 indicate unknown proteins in the pre-transfected human liver cDNA library (BD Biosciences Clontech). pGBK-HTRP and pACT2 containing the Gal4 active domain were used to rule out the possibility that HTRP could turn on the LacZ promoter by itself. pGBKT7-LaminC and pACT-SAP30 were also used to rule out the possibility that SAP30 could turn on the LacZ promoter by itself; pGBKT7-p53 and pGADT7-T were used as positive controls; pGBKT7 and pACT2 were used as negative controls. Yeast colony color was observed after incubation for a standard period of time.

were observed in this work. The quantitative analysis by flow cytometry of fibroblasts at different time points after transfection with HTRP indicated that the expression of HTRP induces cell death compared with control cells transfected with the expression vector pcDNA-3 (Fig. 4). Certainly, no data are presently available to address whether this observation is related the inhibition of specific gene transcription and expression. However, these data imply that the function of HTRP, might involve the repression of some gene related to cell survival.

HTRP Interacting with SAP30 of the mSin3-Protein Complex—In the process of cellular transcriptional regulation, related regulators usually form complexes to bind DNA (*32*, *33*). To investigate the possible function of HTRP, a yeast two-hybrid experiment was performed to explore HTRP-interacting proteins in the cDNA library of human liver tissue. The results suggest that four protein molecules interact with HTRP in cells (Table 1). Of these protein molecules, SAP30 is one component of the human histone deacetylase complex that includes mSin3-protein family members HDAC1 and HDAC2. The other three interacting proteins are unknown, but their genes were identified as being located in human chromosome 4, 7, 12 by the Human Genome Project Working Draft, suggesting that HTRP interacts with SAP30 specifically in the yeast two-hybrid system. In the investigation of the mechanism of the interaction between HTRP and SAP30, 6 deletion mutants of HTRP were produced and reacted with SAP30 in the yeast two-hybrid system (Fig. 5A). Analysis of β-gal activity revealed that HTRP interacts with SAP30 through a domain in the N-terminal region before the 225th amino acid (Fig. 5B), a region containing a conserved sequence showing high homology (53%) with other members of this HTRP protein family. This binding activity reveals that the HTRP molecular interacts with SAP30 in a specific way.

HTRP Interacting with SAP30 and Other Proteins In Vitro and In Vivo—Since the yeast two-hybrid test suggested that HTRP is potentially involved with the co-repressors of the SAP30-mSin3-HDAC complex as described above, a GST pull-down test and Western blotting of the anti-SAP30 antibody were performed to investigate the interaction between HTRP and SAP30 *in vitro*. The result of GST pull-down indicated that three proteins with molecular masses of 60 kDa, 49 kDa and 30 kDa in labeled human embryo fibroblasts are aggregated by GST-HTRP beads but not by GST beads (Fig. 6A), demonstrating that at least three protein molecules are able to interact with HTRP in cells. Further Western blotting indicated that the 30 kDa band was recognized by the anti-SAP30 antibody (Fig. 6B) as confirmed by the interaction of HTRP with SAP30 in cells in the yeast two-

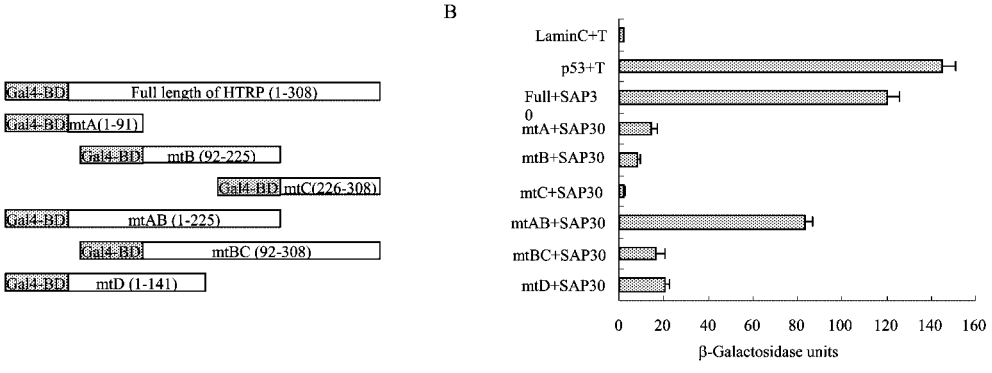

Fig. 5. **Analysis of intrinsic regions of HTRP that interact with SAP30.** A: Schematic drawing of HTRP and its deletion mutants. The plasmids pGBK-mtA, pGBK-mtB, pGBK-mtC, pGBK-mtAB, pGBK-mtBC and pGBK-mtD were cloned by inserting DNA fragments encoding the corresponding peptides from HTRP between the *Eco*RI and XhoI sites of pGBKT7. Grey boxes indicate the DNA-binding domains; mtA (residues 1–91), mtB (residues 92–225), mtC (residues 226–308), mtAB (residues 1–225), mtBC (residues 92–308) and mtD (residues 1–141): 6 deletion mutants of HTRP. B:Transactivation activity of β-Galactosidase when HTRP mutants interact with SAP30. Plasmid pGBKT7 fused each of 6 deletion mutants of HTRP were co-transfected with pACT-SAP30 into yeast AH109 strain. After a freeze-thaw cycle, the yeast cell walls were lysed, and reaction buffer and substrate (ONPG) were added as described. Plasmids pGBKT7-LaminC and pGADT7-T were used as negative controls. Plamids pGBKT7-P53 and pGADT7-T were used as positive controls. The transactivation activity of β-galactosidase was measured at 420 nm, and the β-galactosidase units were calculated according to a standard protocol (Yeast handbook, BD Biosciences Clontech): β-galactosidase units = 1,000 × OD 420 nm/($t \times V \times$ OD 600 nm), where t = elapsed time (in min) of incubation, $V = 0.1 \times$ concentration factor, OD 600 nm = A 600 nm of 1 ml of culture. The values represented the averages of three experiments with variabilities shown by the error bars.

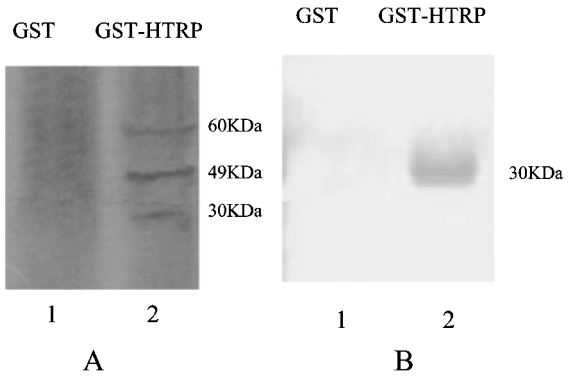

Fig. 6. **Analysis of the interactions of HTRP with SAP30 and other cellular proteins by *in vitro* pull-down assay.** A: Detection of HTRP interactions with SAP30 and other cellular proteins. *In vitro*, ^{35}S-labeled KMB-17 cellular proteins were incubated with equal amounts of GST or GST-HTRP fusion proteins and then immobilized on glutathione–Sepharose 4B beads. Bound proteins were eluted from the beads and separated by 10% SDS-PAGE and analyzed by autoradiography. Lane 1: binding to beads containing GST protein only. Lane 2: labeled KMB-17 cellular extract input GST-HTRP was loaded onto the gel. B: Western blotting Analysis of SAP30 pulled down by the GST-HTRP fusion protein. KMB-17 cellular proteins were incubated with equal amounts of GST or GST-HTRP fusion proteins and then immobilized on glutathione-Sepharose 4B beads. Bound proteins were eluted from the beads and electrophoresed by 10% SDS-PAGE, transferred onto nitrocellulose membranes, and used for Western blotting analysis with an anti-SAP30 polyclonal antibody (Upstate Biotechnology) specific for the designated protein at 1 μg/ml dilution. Signals were detected by an ECL system (Pierce). Lane 1 shows binding to beads containing GST protein only. Lane 2: KMB17 cellular extract input GST-HTRP was loaded onto the gel.

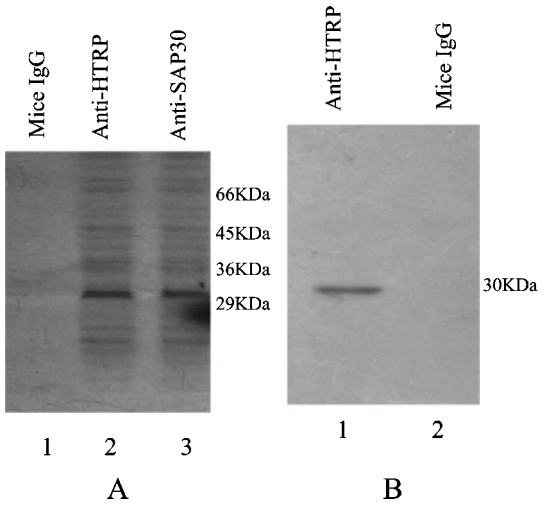

Fig. 7. **The interaction between HTRP and SAP30 *in vivo*.** A: Immunoprecipitation of HTRP from KMB-17 cells bound to HSV1 with anti-HTRP antibody and anti-SAP30 antibody. KMB-17 cells were bound to HSV1 and incubated in [^{35}S]methionine labeling medium, and lysed in RIPA. Immunoprecipitations with antibodies anti-HTRP and anti-SAP30 were performed separately. Then, the conjugated complex was absorbed to A protein-Sepharose 4B. After washing with RIPA, the samples were eluted in sample buffer and subjected to SDS-PAGE. Lane 1 shows negative control with normal mice IgG; Lane 2 shows the complex immunoprecipitated by the anti-HTRP antibody; Lane 3 shows the complex immunoprecipitated by the anti-SAP30 antibody. B: SAP30 was associated with the complex immunoprecipitated by the anti-HTRP antibody. The immunoprecipitated complex aggregated by the anti-HTRP antibody from KMB-17 cells bound to HSV1 as described above was transferred to an NC membrane. Western blotting with anti-SAP30 antibody was performed as described above. Lane 1 shows the specific interaction between the complexes immunoprecipitated by the anti-HTRP antibody and anti-SAP30 antibody; Lane 2 shows the negative control with normal mice IgG.

hybrid test. However, there are sufficient data to interpret whether the other two bands aggregated by the GST-HTRP beads are other components of a possible HTRP complex. They seem to be unknown protein molecules that could be related to transcriptional regulation, especially of a repressors complex based on their interactions with SAP30. Meanwhile, the immunoprecipitation of the anti-HTRP antibody showed that HTRP combines with several protein molecules *in vivo* when expressed in fibroblasts bound to HSV1 (Fig. 7A). These proteins that interact with HTRP were transferred to the NC membrane and Western blotting with the anti-SAP30 antibody was performed, indicating that the SAP30 is included among these proteins (Fig. 7B).

DISCUSSION

Studies on the interaction between virus and receptor suggest that the essence of this interaction actually is a process that mimics the physiological ligand binding receptor and induces signal transduction (*1*, *3*, *5*). With the ability to bind receptors of heparen sulfate moieties and other unidentified membrane proteins, HSV1 is confirmed to be able to induce specific gene responses as it attaches to cells (*8*, *9*). Data from our previous work indicate that the main protein molecules encoded by genes that respond in cells infected with HSV1 are transcriptional regulators (*14*, *18*). As a protein encoded by an immediate-early gene induced in cells bound by HSV1, HTRP was predicted to be related to the transcriptional regulation process through its phosphorylation site, conserved nuclear receptor corepressor motif and a bipartite nuclear localization signal (*35*). Previous analysis of the N-CoR/SMRT complex revealed that a critical motif sequence, L-X-X-X-I-X-X-X-I/L, must form an extended α-helix in the co-repressor in order to fit in the charge clamp of its receptor DNA-binding domain (*20*). This conserved motif is also present in HTRP indicating that HTRP may form a co-repressor complex to repress the DNA-binding domain of some transcriptional activators, or directly inhibit this DNA-binding domain. In fact, HTRP expressed in a fusion polypeptide with a fluorescent protein in HeLa cells was confirmed to localize in the nucleus; also, HTRP was found to be expressed in KMB-17 cells stimulated with HSV1 in our previous work (*16*). Although we do not have detailed data at present to address the precise physiological function of HTRP in cells, the quantitative cytometric analysis of fibroblasts transfected with the htrp gene convinced us to some extent that HTRP might be involved in the repression of some genes related to cell survival. The yeast two-hybrid experiment showed that HTRP is indeed involved in an interaction with the SAP complex via SAP30. The pull-down test and Western blotting also provided evidence to

support this result. The immunoprecipitation of HTRP *in vivo* further confirmed its interaction with SAP30. All data suggest that HTRP is a possible component of a co-repressor of transcriptional regulation. However, since HTRP does not contain a DNA-binding sequence, it could interact indirectly with DNA. As far as we know, co-repressors of transcriptional regulation form several complexes and function in a systematic way, and the SAP30 is able to interact directly with histone deacetylase HDAC1 in its associated function. This ability depends upon protein-protein interactions of multi-components in co-repressor complexes (*28–30*). The observation of mSin3-HDAC-SAP complexes from different laboratories with different compositions implies that the different compositions are probably due to requirements for various regulatory functions (*36*), *e.g*., in TRK2 gene expression, SAP30 is absent from the HDAC/Rpd3 complex (*37*). The interaction between the SAP30 and HTRP induced by the HSV1 binding receptor in the membranes of human embryo fibroblasts suggests a specific functional requirement for signal transduction. In this case, HTRP would likely be a regulator component in a co-repressor complex required for a transcriptional regulatory function, which might be involved in an unknown mechanism to suppress a cellular survival gene.

Some studies have reported composition models of the co-repressor complex (*36*, *38*) depending upon the specific gene expression that is the focus of the research. In the work described here, the HTRP protein induced by the HSV1 binding receptor was found to interact with SAP30, suggesting another possible co-repressor for transcription regulated by a signal activated by the receptor of HSV1 in human embryo fibroblasts. However, this co-repressor is probably part of an unknown mechanism to suppress some cellular survival gene. The fact that HTRP interacts with SAP30 in its conserved domain implies that this protein family, as the product of an immediate-early gene, functions in transcriptional regulation in cells. However, the details of the mechanism for this cellular transcriptional regulation require more investigation.

Nucleotide sequence date are available in the DDBJ/EMBL/GenBank databases under the accession number AF450482.

We thank Mr. Huaisheng Shi for taking photographs for our study. This work was supported by the National Science Funds (No. 30270070), Yunnan Natural Science Funds (No. 2002C0073M) and Specialized Research Fund for the Doctoral Program of Higher Education (SRSDP) (No. 20020023053)

REFERENCES

1. White, J.I. and Wimmer, E.E. (1994) *Cellular Receptors for Animal Virus* pp. 281–301, Colds Spring Harbor Lab Press, Cold Spring Harbor, New York
2. Benn, J. and Schneider, R.J. (1995) Hepatitis B virus HBx protein deregulates cell cycle checkpoint controls. *Proc. Natl Acad. Sci. USA* **92**, 11215–11219
3. Boyle, K.A., Pietropaolo, R.L., and Compton, T. (1999) Engagement of the cellular receptor for glycoprotein B of human cytomegalovirus activates the interferon-responsive pathway. *Mol. Cell. Biol.* **19**, 3607–3613
4. Chang, D.M. and Shaio, M.F. (1994) Production of interleukin-1 (IL-1) and IL-1 inhibitor by human monocytes exposed to dengue virus. *J. Infect. Dis.* **170**, 811–817
5. Mogensen, T.H. and Paludan, S.R. (2001) Molecular pathways in virus-induced cytokine production. *Microbiol. Rev.* **65**, 131–150
6. Tamanini, A., Rolfini, R., Nicolis, E., Melotti, P., and Cabrini, G. (2003) MAP kinases and NF-kappaB collaborate to induce ICAM-1 gene expression in the early phase of adenovirus infection. *Virology* **307**, 228–242
7. Shen, H., Cheng, T., Preffer, F.I., Dombkowski, D., Tomasson, M.H., Golan, D.E., Yang, O., Hofmann, W., Sodroski, J.G., Luster, A.D., and Scadden, D.T. (1999) Intrinsic human immunodeficiency virus type 1 resistance of hematopoietic stem cells despite coreceptor expression. *J. Virol.* **73**, 728–737
8. Fields, B.N., Knipe, D.M., and Howley, P.M. (1996) Herpes Simplex virus and their replication in *Fields Virology* pp.1658–1765, Lippincatt-Raven, Philadelphia
9. Halford, W.P. and Carr, D.J. (1995) Subversion of intracellular signal transduction by herpes simplex virus type 1. *Adv. Neuroimmunol.* **5**, 327–334
10. Chou, J. and Roizman, B. (1992) The gamma 1(34.5) gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programed cell death in neuronal cells. *Proc. Natl Acad. Sci. USA* **89**, 3266–3270
11. Tal-Singer, R., Podrzucki, W., Lasner, T.M., Skokotas, A., Leary, J.J., Fraser, N.W., and Berger, S.L. (1998) Use of differential display reverse transcription-PCR to reveal cellular changes during stimuli that result in herpes simplex virus type 1 reactivation from latency: upregulation of immediatee-early cellular response genes TIS7, interferon, and interferon regulatory factor-1. *J. Virol.* **72**, 1252–1261
12. Mossman, K.L., Macgregor, P.F., Rozmus, J.J., Goryachev, A.B., Edwards, A.M., and Smiley, J.R. (2001) Herpes simplex virus triggers and then disarms a host antiviral response. *J. Virol.* **75**, 750–758
13. Leopardi, R., Van Sant, C., and Roizman, B. (1997) The herpes simplex virus 1 protein kinase US3 is required for protection from apoptosis induced by the virus. *Proc. Natl Acad. Sci. USA* **94**, 7891–7896
14. Li, Q.H., Zhao, H.L., Jiang, L., Che, Y.C., Dong, C.H., Wang, L.C., and Liu, L.D. (2002) A SR protein induced by HSV1 bingding to cells functioning as a splicing inhibitor of viral preмRNA. *J. Mol. Biol.* **316**, 887–894
15. Li, Q.H., Dong, C.H., Wang, L.C., Sun, M., and Yang, F. (2000) Early expression of protooncogenes induced by the binding to cells of two viruses. *Acta Biochim. Biophys.* **32**, 149–152
16. Liu, L.D., Dong, C.H., Dong, S.Z., and Li, Q.H. (2003) Cloning, Expression and Purification of HTRP Gene from KMB-17 cell induced by HSV1. *Chin. J. Virol.* **19**, 16–20
17. Wang, J., Liu, L.D., Dong, C.H., Sun, M., and Li, Q.H. (2002) Cloning and characterization of PEBP-like gene. *Virologica Sinica* **17**, 97–101
18. Dong, S.Z., Dong, C.H., Liu, L.D., Che, Y.C., Sun, M., Hu, F., Li, J.F., and Li, Q.H. (2003) Identification of a novel human sand family protein in human fibroblasts induced by Herpes simplex virus 1 binding. *Acta Virol.* **47**, 27–32
19. Burke, L.J. and Baniahamad, A. (2000) Co-repressors 2000. *FASEB J.* **14**, 1876–1888
20. Jepsen, K. and Rosenfeld, M.G. (2002) Biological roles and mechanistic actions of co-repressor complexes. *J. Cell Sci.* **115**, 689–698
21. Wolffe, A.P., Urnov, F.D., and Guschin, D. (2000) Co-repressor complexes and remodelling chromatin for repression. *Biochem. Soc. Trans* **28**, 379–386
22. Caron, C., Pivot-Pajot, C., van Grunsven, L.A., Col, E., Lestrat, C., Rousseaux, S., and Khochbin, S. (2003) Cdy1:a new transcriptional co-repressor. *EMBO Rep.* **4**, 877–882
23. Jung, M. (2001) Inhibitors of histone deacetylase as new anticancer agents. *Curr. Med. Chem.* **8**, 1505–1511
24. David, G., Alland, L., Hong, S.H., Wong, C.W., DePinho, R.A., and Dejean, A. (1998) Histone deacetylase associated with mSin3A mediates repression by the acute promyelocytic leukemia-associated PLZF protein. *Oncogene* **16**, 2549–2556

25. Kaczynski, J., Zhang, J.S., Ellenrieder, V., Conley, A., Duenes, T., Kester, H., van Der Burg, B., and Urrutia, R. (2001) The Sp1-like protein BTEB3 inhibits transcription via the basic transcription element box by interacting with mSin3A and HDAC-1 co-repressors and competing with Sp1. *J. Biol. Chem.* **276**, 36749–36756
26. Alland, L., David, G., Shen-Li, H., Potes, J., Muhle, R., Lee, H.C., Hou, H.Jr., Chen, K., and DePinho, R.A. (2002) Identification of mammalian Sds3 as an integral component of the Sin3/histone deacetylase corepressor complex. *Mol. Cell. Biol.* **22**, 2743–2750
27. Zhang, Y., Sun, Z.W., Iratni, R., Erdjument, B.H., Tempst, P., Hampsey, M., and Reinberg, D. (1998) SAP30, a novel protein conserved between human and yeast, is a component of a histone deacetylase complex. *Mol. Cell* **1**, 1021–1031
28. Huang, E.Y., Zhang, J., Miska, E.A., Guenther, M.G., Kouzarides, T., and Lazar, M.A. (2000) Nuclear receptor corepressors partner with class II histone deacetylases in a Sin3-independent repression pathway. *Genes Dev.* **14**, 45–54
29. Silverstein, R.A., Richardson, W., Levin, H., Allshire, R., and Ekwall, K. (2003) A new role for the transcriptional corepressor SIN3; regulation of centromeres. *Curr. Biol.* **13**, 68–72
30. Bernstein, B.E., Tong, J.K., and Schreiber, S.L. (2000) Genomewide studies of histone deacetylase function in yeast. *Proc. Natl Acad. Sci. USA* **97**, 13708–13713
31. Li, J.W., Lin, Q.S., Wang, W.D., Paul, W., and Wong, J.M. (2002) Specific targeting and constitutive association of histone deacetylase complexes during transcriptional repression. *Genes Dev.* **16**, 687–692
32. Wadkins, R.M. (2000) Targeting DNA secondary structures. *Curr. Med. Chem.* **7**, 1–15
33. Boyd, K.E. and Farnham, P.J. (1999) Identification of target genes of oncogenic transcription factors. *Proc. Soc. Exp. Biol. Med.* **222**, 9–28
34. Melchjorsen, J., Pedersen, F.S., Mogensen, S.C., and Paludan, S.R. (2002) Herpes Simplex Virus Selectively Induces Expression of the CC Chemokine RANTES/CCL5 in Macrophages through a Mechanism Dependent on PKR and ICP0. *J. Virol.* **76**, 2780–788
35. Robbins, J., Dilworth, S.M., and Laskey, R.A. (1991) Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. *Cell* **64**, 615–623
36. Fleischer, T.C., Yun, U.J., and Ayer, D.E. (2003) Identification and characterization of three new components of the mSin3A corepressor complex. *Mol. Cell. Biol.* **23**, 3456–3467
37. Vannier, D., Balderes, D., and Shore, D. (1996) Evidence that the transcriptional regulators SIN3 and RPD3, and a novel gene (SDS3) with similar functions, are involved in transcriptional silencing in S. cerevisiae. *Genetics* **144**, 1343–1353
38. Shi, Y., Sawada, J., Sui, G., Affar el, B., Whetstine, J.R., Lan, F., Ogawa, H., Luke, M.P., Nakatani, Y., and Shi, Y. (2003) Coordinated histone modifications mediated by a CtBP corepressor complex. *Nature* **422**, 735–738.