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Molecular Cloning of a Novel Human I-mfa Domain-containing Protein That Differently Regulates Human T-cell Leukemia Virus Type I and HIV-1 Expression*

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Regulation of viral genome expression is the result of complex cooperation between viral proteins and host cell factors. We report here the characterization of a novel cellular factor sharing homology with the specific cysteine-rich C-terminal domain of the basic helix-loop-helix repressor protein I-mfa. The synthesis of this new factor, called HIC for Human I-mfa domain-Containing protein, is controlled at the translational level by two different codons, an ATG and an upstream non-ATG translational initiator, allowing the production of two protein isoforms, p32 and p40, respectively. We show that the HIC protein isoforms present different subcellular localizations, p32 being mainly distributed throughout the cytoplasm, whereas p40 is targeted to the nucleolus. Moreover, in trying to understand the function of HIC, we have found that both isoforms stimulate in T-cells the expression of a luciferase reporter gene driven by the human T-cell leukemia virus type I-long terminal repeat in the presence of the viral transactivator Tax. We demonstrate by mutagenesis that the I-mfa-like domain of HIC is involved in this regulation. Finally, we also show that HIC is able to down-regulate the luciferase expression from the human immunodeficiency virus type 1-long terminal repeat induced by the viral transactivator Tat. From these results, we propose that HIC and I-mfa represent two members of a new family of proteins regulating gene expression and characterized by a particular cysteine-rich C-terminal domain.

Human T-cell leukemia virus type I (HTLV-I)[1] and human immunodeficiency virus type 1 (HIV-1) are both human retroviruses that infect *in vivo* $CD4^+$ T lymphocytes. However, these infections lead to two extremely different diseases. Infection with HTLV-I results in dysregulation of T-cell proliferation, sometimes causing the development of adult T-cell leukemia. By contrast, infection with HIV-1 causes the loss of T-cells, resulting in systemic immunosuppression and development of AIDS. Characterization of molecular mechanisms involved in the interactions between viral and cellular components contributes to understanding why HTLV-I and HIV-1 have different effects on the infected T-cell growth. For instance, the transcriptional regulators used by T-cells to control cell function are used differently by HTLV-I and HIV-1 to regulate expression of their genome. Both retroviruses utilize the cellular RNA polymerase II to transcribe their genome (1–4) but also code for their own regulatory proteins that regulate the transcription of viral and cellular genes. Thus, HIV-1 Tat protein is able to activate transcription from the viral long terminal repeat (LTR) promoter (5–7) by interacting with the transactivation responsive element located at the 5′ end of viral mRNAs (8–10) and then with general transcription factors (for reviews see Refs. 11 and 12). By contrast, HTLV-I Tax protein is unable to bind specifically to nucleic acids (13–15). To stimulate transcription from LTR promoter, Tax is recruited by interaction with the activating transcription factors/CRE-binding proteins (ATF/CREB) (16–20) that bind to the three imperfect 21-bp cAMP-responsive elements located in the U3 of the LTR (21–25). Then, Tax could stimulate transcription by recruiting the coactivator CREB-binding protein (CBP) (26–28) and by interacting with basal transcription factors (29–31).

Basic helix-loop-helix (bHLH) proteins regulate cell determination and differentiation during embryogenesis (for reviews see Refs. 32 and 33). For example, the cell fate of skeletal muscle precursors is regulated by the *MyoD* subfamily of bHLH factors including *MyoD*, *Myf5*, myogenin, and *MRF4* (33, 34). bHLH proteins are also involved in lineage commitment and differentiation as Mash2 and Hand1, which play a role in trophoblast development (35–37). The activity of bHLH factors is regulated to achieve the coordinated expression of genes during development. Recently, a novel myogenic repressor has been identified called I-mfa for Inhibitor of *MyoD* family (38, 39). I-mfa is generated with two additional proteins, I-mfb and I-mfc, by alternative splicing (38). The three I-mf proteins share a common N-terminal region, but each has a different C terminus, the I-mfa-specific C terminus being characterized by a high content of cysteines. I-mfa is distributed mainly throughout the cytoplasm, although it is also detectable in the nucleus (38). I-mfa inhibits myogenic bHLH proteins by retaining them in the cytoplasm and by interfering with their DNA binding activity in the nucleus (38). I-mfa is also able to inhibit

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF054589.

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[1] The abbreviations used are: HTLV-I, human T-cell leukemia virus type I; HIV-1, human immunodeficiency virus type 1; bp, base pair; LTR, long terminal repeat; CREB, CRE-binding proteins; CBP, CREB-binding protein; bHLH, basic helix-loop-helix; PAGE, polyacrylamide gel electrophoresis; GFP, green fluorescent protein.

the transactivation activity of Mash2 and plays an important role in trophoblast and chondrogenic differentiation (39). On the other hand, the functions of I-mfb and I-mfc still remain unclear.

In this study, we report the isolation and characterization of a human cDNA clone encoding a novel protein, which exists in two isoforms differing by the presence or absence of a basic amino acid-rich N-terminal domain and by their localization in the cell. Both isoforms contain a common C terminus sharing homology with the specific C-terminal domain of I-mfa. We show that this new factor, called HIC for human I-mfa domain-containing protein, stimulates the expression of a luciferase reporter gene driven by the HTLV-I LTR in the presence of Tax. By using mutagenesis, we demonstrate that the I-mfa-like domain of HIC is required to stimulate luciferase expression. Finally, we also show that HIC is able to down-regulate expression from HIV-1 LTR in the presence of Tat. From these results, we propose that HIC and I-mfa represent a new family of proteins regulating gene expression and are characterized by the presence of a specific cysteine-rich C-terminal domain. Our results also suggest that HIC could differently regulate expression of HTLV-I and HIV-1 genomes.

EXPERIMENTAL PROCEDURES

Molecular Cloning of a 4,152-bp Full-length HIC cDNA—HIC cDNA clones were first isolated from a MT-2 cDNA library by the yeast two-hybrid approach. MT-2 cDNA fused to the GAL4 activation domain of the pGAD10 vector (20) was screened by using the cytoplasmic tail of CD4 as a bait fused to the LEXA DNA binding domain of the pBTM116 vector. The two-hybrid screen was performed as already described (20) with the *Saccharomyces cerevisiae* L40 reporter strain.

The multiple tissue Northern blot and the human spleen cDNA library cloned into λgt11 were purchased from CLONTECH. RNA hybridization and library screening were performed as described by the manufacturer. The HIC cDNA encompassing the nucleotides from position 1148 to 1639 was labeled with [α-^{32}P]dCTP using the method of random priming and was used as a probe.

In Vitro Transcription and Translation—All the different HIC cDNAs cloned into pSPORT 1 (Life Technologies, Inc.) were transcribed and translated in the presence of [^{35}S]methionine and [^{35}S]cysteine by using the TNT T7 coupled transcription-translation reticulocyte lysate system of Promega. Translation products were analyzed by SDS-PAGE and autoradiography. pHIC-1 corresponds to an *Sal*I-*Spe*I fragment that contains the first 1532 bp of the HIC cDNA subcloned into pSPORT 1. The 5′-deleted plasmids, pHIC-2, -3, -4, and -5, were either constructed by restriction endonuclease digestion and religation of pHIC-1 or generated by PCR amplification on pHIC-1 and subcloned into pSPORT 1. Templates where CTG or GTG were mutated were also generated by PCR amplification; CTG at position 246 was transformed into ATG (pHIC-I-atg) or CGG (pHIC-I-cgg), GTG at position 264 into ATG (pHIC-II-atg) or CGG (pHIC-II-cgg), and CTG at position 321 into ATG (pHIC-III-atg).

Expression of HIC p32 and p40 Tagged with Either GFP or myc Epitope in COS7 Cells—To express HIC p32 and p40 with a GFP or *myc* tag, the complete coding sequences of both proteins were subcloned into the vectors pEGFP-N1 (CLONTECH), pEGFP-C1 (CLONTECH), and pcDNA3.1(−)/Myc-His (Invitrogen). We also cloned an *Nhe*I-*Kpn*I fragment of HIC cDNA (from position 95 to 1328) into pEGFP-N1 to analyze the production of HIC protein isoforms *in vivo* from the wild type leader. COS7 cells were transfected using the calcium phosphate-mediated transfection method with 20 μg of expression vector. Cells were cultivated on the glass slides and then analyzed by fluorescence 24 h after transfection. p32 and p40 tagged with the *myc* epitope were detected by using the anti-*myc* monoclonal antibody purchased from Sigma and goat anti-mouse immunoglobin G antibody coupled to fluorescein isothiocyanate. Analysis of the green, red, and yellow fluorescence was performed with a Bio-Rad MRC 1024 confocal microscope.

Transfections and Luciferase Assays—The coding sequences of p32 and p40 were cloned into a eukaryotic expression vector, pcDNA3.1/His (Invitrogen). The plasmid pcDNA3.1/His-HICΔ, which contains the entire coding sequence of p40 except for the I-mfa-like domain, was constructed by digesting p40 cDNA cloned into pcDNA3.1/His with *Eco*RV and *Xho*I. The resulting digest was treated with Klenow and religated as blunt ends. This approach resulted in the deletion of the last 101 amino acids. The Tat and Tax expression vectors, pBg312HIV-1Lai-Tat and pSG-Tax, respectively, have been described previously (40, 41). CEM cells were transiently cotransfected according to the procedure published previously (42). 5 μg of pACβ1 (β-galactosidase-containing reference plasmid) was included in each transfection for controlling the transfection efficiency. The total amount of DNA in each transfection was the same, the balance being made up with empty pcDNA3.1/His. Cell extracts equalized for protein content were used for luciferase and β-galactosidase assays. For the assays with the GAL4-binding site promoter-reporter plasmid, HIC (amino acids 120 to 355), Tax, and Tat were fused in frame with the DNA-binding domain of GAL4 (cloned into pBIND vector, Promega). Cotransfection assays were performed in CEM cells in the presence of the luciferase reporter plasmid pG5luc containing five GAL4-binding sites upstream of a minimal TATA box.

Immunoprecipitation and Western Blot Assays—Protein extracts were electrophoresed onto SDS-10% polyacrylamide gel (SDS-10% PAGE) and blotted to polyvinylidene difluoride membranes (Millipore). The blot was then incubated overnight at 4 °C with a blocking solution (phosphate-buffered saline containing 5% milk) prior to addition of antiserum. After 2 h at 20 °C, the blot was washed three times with 0.5% phosphate-buffered saline/Tween 20 and incubated for 2 h with goat anti-mouse or anti-rabbit immunoglobulin-peroxidase conjugate (Immunotech, Marseille, France). After three washes, the membrane was incubated with enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech). The membrane was then exposed for 0.5 to 5 min to hyperfilms-ECL (Amersham Pharmacia Biotech). The anti-Xpress serum was purchased from Invitrogen and recognizes the tag found in the Xpress leader peptide in the vector pcDNA3.1/His. The anti-HIC serum was obtained by immunizing rabbits with purified His_6-tagged HIC polypeptide corresponding to the first 163 amino acids of p32. HIC cDNA was cloned into the bacterial expression vector pQE-30 (Qiagen). The N-terminal His_6-tagged protein was purified as described by the manufacturer. Immunoprecipitation assay was carried out as described previously (43).

RESULTS

Isolation of HIC cDNA, Which Encodes a Protein Sharing Homology with the Specific C-terminal Domain of I-mfa—Two cDNA clones coding, respectively, for the last 60 and 153 C-terminal amino acids of a novel protein were isolated from an HTLV-I-infected T-cell line cDNA library (20). As the predicted polypeptide presented homologies with the specific C-terminal domain of I-mfa, a cellular factor known to be a bHLH repressor, we decided to characterize further this novel protein. At first, the tissue distribution of the mRNA coding for this novel HIC protein was analyzed. All the tested lymphoid organs (spleen, thymus, and peripheral blood leukocytes) expressed an mRNA of about 4.4 kb (Fig. 1). This mRNA is not specific to lymphoid tissues since it is expressed in prostate, uterus, and small intestine. Finally, it is almost absent in testis and colon. From these observations, we decided to screen a human spleen cDNA library cloned into λgt11 to characterize the complete sequence of HIC cDNA. By this approach, we were able to isolate a 4,152-bp full-length HIC cDNA that was completely sequenced (GenBank™ number AF054589).

Mapping of the Translation Initiation Sites—The full-length HIC cDNA contains an open reading frame encoding a polypeptide of 246 amino acids if the ATG at position 591 is the initiation codon (Fig. 2). We examined the proteins synthesized in a cell-free system with a cDNA containing the first 1532 nucleotides of HIC sequence (the stop codon TAA is at position 1329; see Fig. 2). SDS-PAGE of the translational products effectively revealed a protein of 32 kDa, p32, but also an unexpected product of 40 kDa, p40 (Fig. 3*a, lane pHIC-1*). To determine the origin of both polypeptides, a series of 5′ truncation mutants of pHIC-1 was generated. These experiments (Fig. 3) demonstrated that p32 translation initiation was located between position 393 and 617 (compare the *lanes pHIC-3* and *pHIC-5*) suggesting that ATG at position 591 could be an initiation codon. To confirm this hypothesis, the entire region 5′ to this ATG was deleted. SDS-PAGE of the translation products

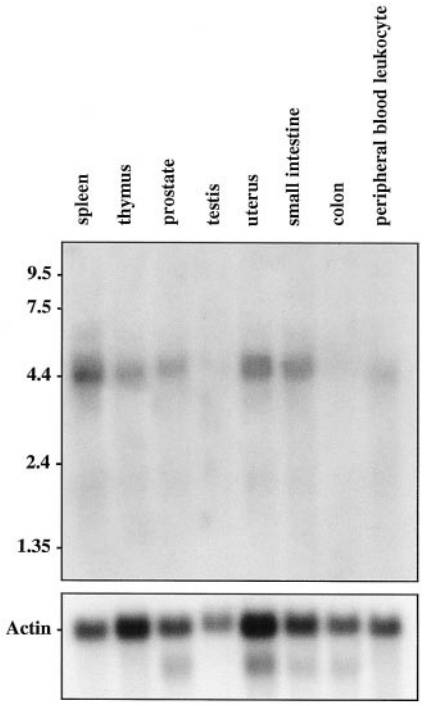

FIG. 1. **Tissue distribution of HIC mRNA.** Northern blot of poly(A)$^+$ RNA from various human tissues was analyzed. Molecular size markers in kilobases (*kb*) are shown on the *left*.

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CCCAGGCCGGCTCTGGCCTCCTGACCCAGACAGCGCAGGGCGCGAGGGATCGCGCGCGCCG 60
AGCCCGGGTCGCGCCGCTCCCAGCATCGGGGCCGCTAGCCAAGAGTTCGAGGCCTTCCCG 120
ATCCGGATGTGATGAAAAAGAGCAACAGAGGGAGAAGTGTTTCAGGATTGTAGGAGTGGA 180
                                                      E  W

AGAGGGGAAAGAGAGGCAGAGAGGGGGAAGGCCCCCTCGCAGGGGAGCCGGCTGGAGTGA 240
K  R  G  K  R  G  R  E  G  E  G  L  L  A  G  E  P  A  G  V

GCTGGCTGGAAAGAGGGGGCGGAGTGCGCGGAGTCAGAGCCGCCACCGCTGCCGCAGTTG 300
S  W  L  E  R  G  G  G  V  R  G  V  R  A  A  T  A  A  A  V

CCGCCACTGCGGCGTCTGGGCTGAGCCGGAGGGAGGCGGGAGGACGCGCAGGGGCGGCCG 360
A  A  T  A  A  S  G  L  S  R  R  E  A  G  G  R  A  G  A  A

CCGCCGTCGTCAGGCCACCGGGGCGAAAATGCGGCCGCTGCCGGAGGCTCGCTAACTTTC 420
A  A  V  V  R  P  P  G  R  K  C  G  R  C  R  R  L  A  N  F

CGGGGCGGAAGAGGAGGAGGAGGAGGAGGAAGGGGCTTGGAGCGACTACGGGGGGATGCG 480
P  G  R  K  R  R  R  R  R  R  K  G  L  G  A  T  T  G  G  C

GAGAAGCAGTCAGTTCCCTGCACCCAGCACCTCACAGCCCTTCCTCCGTGCGCCCTGCCG 540
G  E  A  V  S  S  L  H  P  A  P  H  S  P  S  S  V  R  P  A

GGCGGCGAGCTAGGCGGCAGCGGCGCGGCGCGGGCTCGGCGGAGCGGCCCATGTCCGGCG 600
G  R  R  A  R  R  Q  R  R  G  A  G  S  A  E  R  P  M  S  G

CGGGCGAAGCCCTCGCTCCCGGGCCCGTGGGGCCGCAGCGCGTGGCCGAGGCGGGCGGCG 660
A  G  E  A  L  A  P  G  P  V  G  P  Q  R  V  A  E  A  G  G

GCCAGCTGGGCTCCACAGCCCAGGGAAAATGTGATAAAGACAATACTGAGAAAGATATAA 720
G  Q  L  G  S  T  A  Q  G  K  C  D  K  D  N  T  E  K  D  I

CTCAAGCTACCAATAGCCACTTCACACATGGAGAGATGCAAGACCAGTCCATTTGGGGAA 780
T  Q  A  T  N  S  H  F  T  H  G  E  M  Q  D  Q  S  I  W  G

ATCCTTCGGATGGTGAACTCATTAGAACCCAACCTCAGCGCTTGCCTCAGCTTCAGACTT 840
N  P  S  D  G  E  L  I  R  T  Q  P  Q  R  L  P  Q  L  Q  T

CAGCACAGGTGCCAAGTGGTGAGGAAATAGGCAAGATAAAGAACGGCCACACAGGTCTGA 900
S  A  Q  V  P  S  G  E  E  I  G  K  I  K  N  G  H  T  G  L

GCAATGGAAATGGAATTCACCACGGGGCCAAACACGGATCCGCAGATAATCGCAAACTTT 960
S  N  G  N  G  I  H  H  G  A  K  H  G  S  A  D  N  R  K  L

CAGCACCTGTTTCTCAAAAAATGCATAGAAAAATTCAGTCCAGCTTGTCTGTAAACAGCG 1020
S  A  P  V  S  Q  K  M  H  R  K  I  Q  S  S  L  S  V  N  S

ATATCAGTAAGAAGAGCAAAGTAAATGCTGTCTTTTCCCAAAAGACAGGCTCTTCACCTG 1080
D  I  S  K  K  S  K  V  N  A  V  F  S  Q  K  T  G  S  S  P

AAGATTGTTGTGTCCACTGTATCCTGGCTTGCTTGTTCTGCGAATTCCTGACCCTTTGCA 1140
E  D  C  C  V  H  C  I  L  A  C  L  F  C  E  F  L  T  L  C

ACATTGTCCTGGGACAAGCGTCATGTGGCATCTGCACCTCAGAAGCCTGCTGCTGTTGCT 1200
N  I  V  L  G  Q  A  S  C  G  I  C  T  S  E  A  C  C  C  C

GTGGTGACGAGATGGGGGATGATTGTAACTGCCCTTGTGATATGGACTGTGGCATCATGG 1260
C  G  D  E  M  G  D  D  C  N  C  P  C  D  M  D  C  G  I  M

ATGCCTGTTGTGAATCATCAGACTGCTTGGAAATCTGTATGGAATGCTGTGGAATTTGTT 1320
D  A  C  C  E  S  S  D  C  L  E  I  C  M  E  C  C  G  I  C

TTCCTTCATAA 1331
F  P  S
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FIG. 2. **The 5′ end nucleotide and deduced amino acid sequences of the full-length HIC cDNA isolated from a human spleen cDNA library.** The amino acid sequence deduced from the longest open reading frame is shown *below* the nucleotides. The sequence data of the complete HIC cDNA have been submitted to the GenBank™ data base under accession number AF054589.

synthesized from this template revealed one major protein of 32 kDa (lane pHIC-4) indicating that the first ATG effectively is the initiation codon involved in p32 synthesis.

On the other hand, from the template pHIC-3, p40 was no longer synthesized, whereas it was still produced from pHIC-2 (Fig. 3*a*). This observation suggests that the HIC cDNA clone contains another initiation site upstream of the first ATG, located between positions 174 and 393. There are many examples of proteins where codons other than ATG are initiation codons (44–46). In human cells, TTG and CTG have been found as initiation codons. To determine whether one or both CTG located upstream of the ATG (Fig. 3*b*) could be translational initiator, templates starting at positions 246 or 321, where CTG was transformed into ATG (respectively, pHIC-I-atg and pHIC-III-atg), were constructed. Only the ATG which starts at position 246 generated a 40-kDa major product that could correspond to p40 (Fig. 3*a, lane pHIC-I-atg*). However, when the CTG at position 246 was mutated into a non-initiation codon (CGG), p40 was still synthesized (Fig. 3*a, lane pHIC-I-cgg*). Based on these results, it appears that both CTGs are not initiation codons and that the initiation codon must be contained within the nucleotide region between position 246 and 321. In this region, only the GTG located at position 264 belongs to the non-ATG codons known to be able to initiate translation in mammalian cells. For this reason, this GTG was mutated into ATG or CGG corresponding, respectively, to the plasmids pHIC-II-atg and pHIC-II-cgg (Fig. 3*b*). From pHIC-II-atg a 40-kDa protein was produced, whereas the synthesis of this product was abolished with pHIC-II-cgg (Fig. 3*a*). This result clearly demonstrates that the GTG at position 264 is initiator in our cell-free system.

Taken together, our data clearly demonstrate that HIC mRNA codes for two protein isoforms, p32 and p40, synthesized from two different initiation codons as follows: a standard ATG initiator for p32, and a GTG located upstream of the ATG for p40. p32 and p40 only differ by the presence of a N-terminal sequence containing two basic subdomains (Fig. 4*a*). Moreover, their common C-terminal region shares 77% identical amino acids with the specific C-terminal domain of I-mfa (Fig. 4*b*).

Localization of HIC p32 and p40 in Vivo—Next we investigated the subcellular localization of both HIC protein isoforms *in vivo*. COS7 cells were transfected with vectors expressing p32 or p40 tagged with green fluorescent protein (GFP), fused either to their N-terminal end or their C-terminal end. As shown in Fig. 5, the position of the GFP tag apparently has no influence on the localization of the HIC proteins. p32 is distributed primarily throughout the cytoplasm, although weak staining is detectable in the nucleus (Fig. 5, *a* and *c*). Two different cytoplasmic patterns of p32 localization are observed, a diffuse (Fig. 5, *a* and *c*) and a bright punctate staining (Fig. 5, *b* and *d*). p40 also exhibits a granular distribution in the cytoplasm, but in addition, it shows a staining pattern in the nucleus, localized around and in the nucleoli (Fig. 5, *e* and *f*).

To be certain that the GFP tag did not influence their localization, p32 and p40 were fused to a smaller tag, containing either the *myc* epitope fused to their C-terminal end (Fig. 6) or the X-press leader peptide fused to their N-terminal end (data not shown). In COS7 cells transfected with these constructs, p32 and p40 give the same staining pattern as that observed with the GFP tag (compare Fig. 5 and 6). In conclusion, p32 is

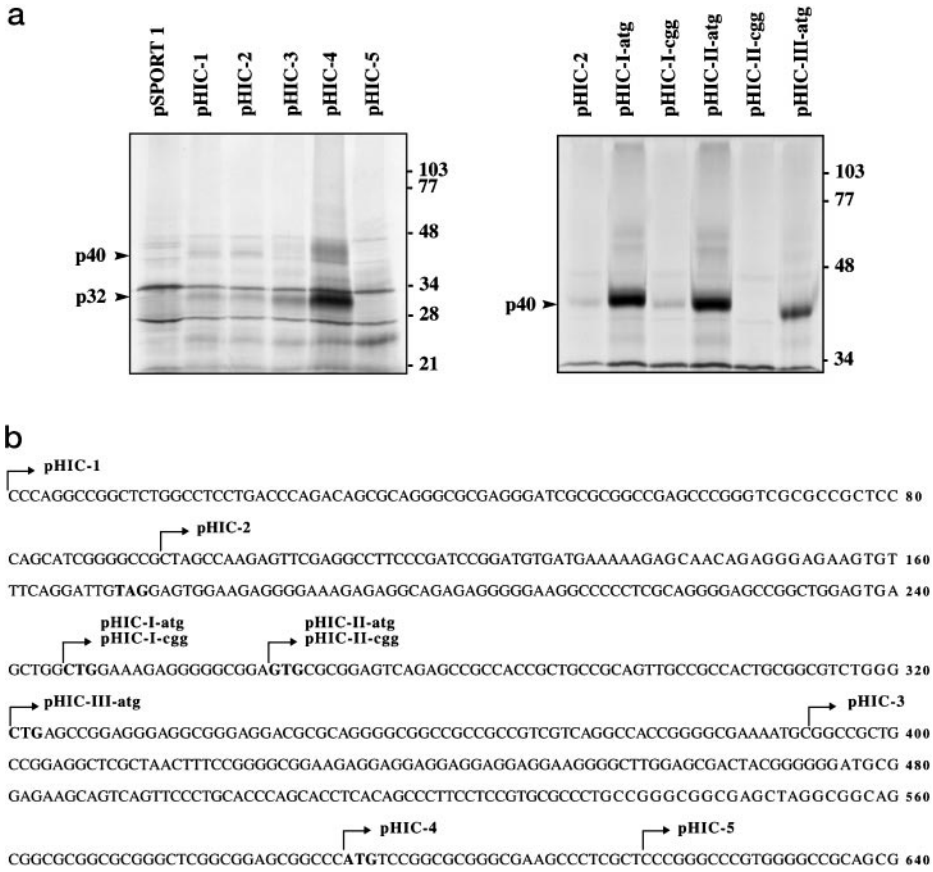

FIG. 3. **Mapping of the translation initiation sites of the HIC cDNA clone with a cell-free system.** *a*, all mRNAs were translated in rabbit reticulocyte lysates with either an HIC cDNA clone containing the first 1532 bp or PCR-generated DNAs as templates for transcription. [^{35}S]Methionine and [^{35}S]cysteine were used in translation reactions to label proteins. Translation products were analyzed by SDS-PAGE and autoradiography. ^{35}S-Labeled p40 and p32 are designated by *arrows*. Molecular size markers in kilodaltons are shown on the *right*. The exact start of the different HIC constructs are shown in *b below* the autoradiographs. *b*, nucleic acid sequence of the 5′ end of the HIC cDNA clone. The stop codon TAG at position 171 and the putative initiation codons are indicated in *bold*. The exact position of the 5′ end of the different HIC cDNA clones is indicated by an *arrow*. pHIC-1 and the 5′ deleted plasmids, pHIC-2, -3, -4, and -5, encode the wild type nucleotide sequence of HIC cDNA. For the other deleted constructs, the 5′ end was modified as follows: CTG at position 246 was transformed into ATG (pHIC-I-atg) or CGG (pHIC-I-cgg), GTG at position 264 into ATG (pHIC-II-atg) or CGG (pHIC-II-cgg), and CTG at position 321 into ATG (pHIC-III-atg).

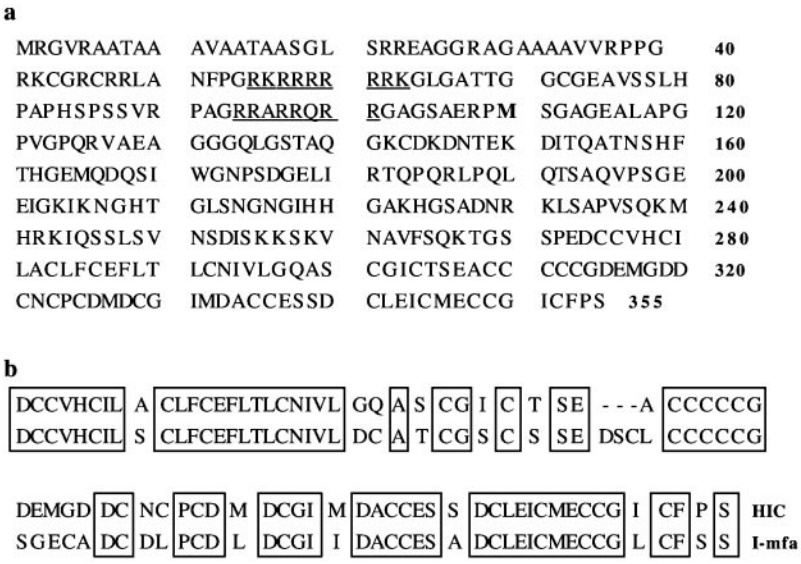

FIG. 4. **The amino acid sequence of HIC p32 and p40.** *a*, the complete amino acid sequence corresponds to HIC p40. *Underlined* amino acids delineate both basic rich subdomains of p40. The first methionine of p32 corresponding to initiation codon is indicated in *bold*. *b*, the C-terminal 82 amino acids of HIC protein aligned with the specific C-terminal domain of I-mfa. Identical residues are *boxed*.

predominantly cytoplasmic, whereas p40 is both cytoplasmic and nuclear. Moreover, p32 is distributed in both a diffuse and a punctate staining pattern throughout the cytoplasm. We do not know why two different patterns are observed with p32. However, preliminary data[2] suggest that the I-mfa-like domain of HIC could be involved in the formation of these cytoplasmic granular structures.

Expression of HIC p32 and p40 in Vivo—To raise polyclonal antibodies against HIC, rabbits were immunized with highly purified HIC polypeptide corresponding to the first 163 amino acids of p32. We studied the expression of HIC protein isoforms *in vivo* with this antiserum by using two additional approaches. First, we analyzed by Western blotting (Fig. 7*a*) protein extracts of 293T cells transfected with the eukaryotic expression vector pcDNA3.1(−)/Myc-His containing the HIC coding sequence under the control of the wild type leader (from nucleotide 95 to 1300, see Fig. 2). Second, we performed immunoprecipitation with anti-HIC from extracts of HTLV-I infected T-cells (Fig. 7*b*). In both cases, a single protein was detected with anti-HIC but not with preimmune serum (Fig. 7), the size of this protein being consistent with the size of recombinant p32 when expressed in eukaryotic cell lines (data not shown). These results demonstrate that p32 is the major protein isoform produced from HIC mRNA *in vivo*.

[2] S. Thébault, unpublished results.

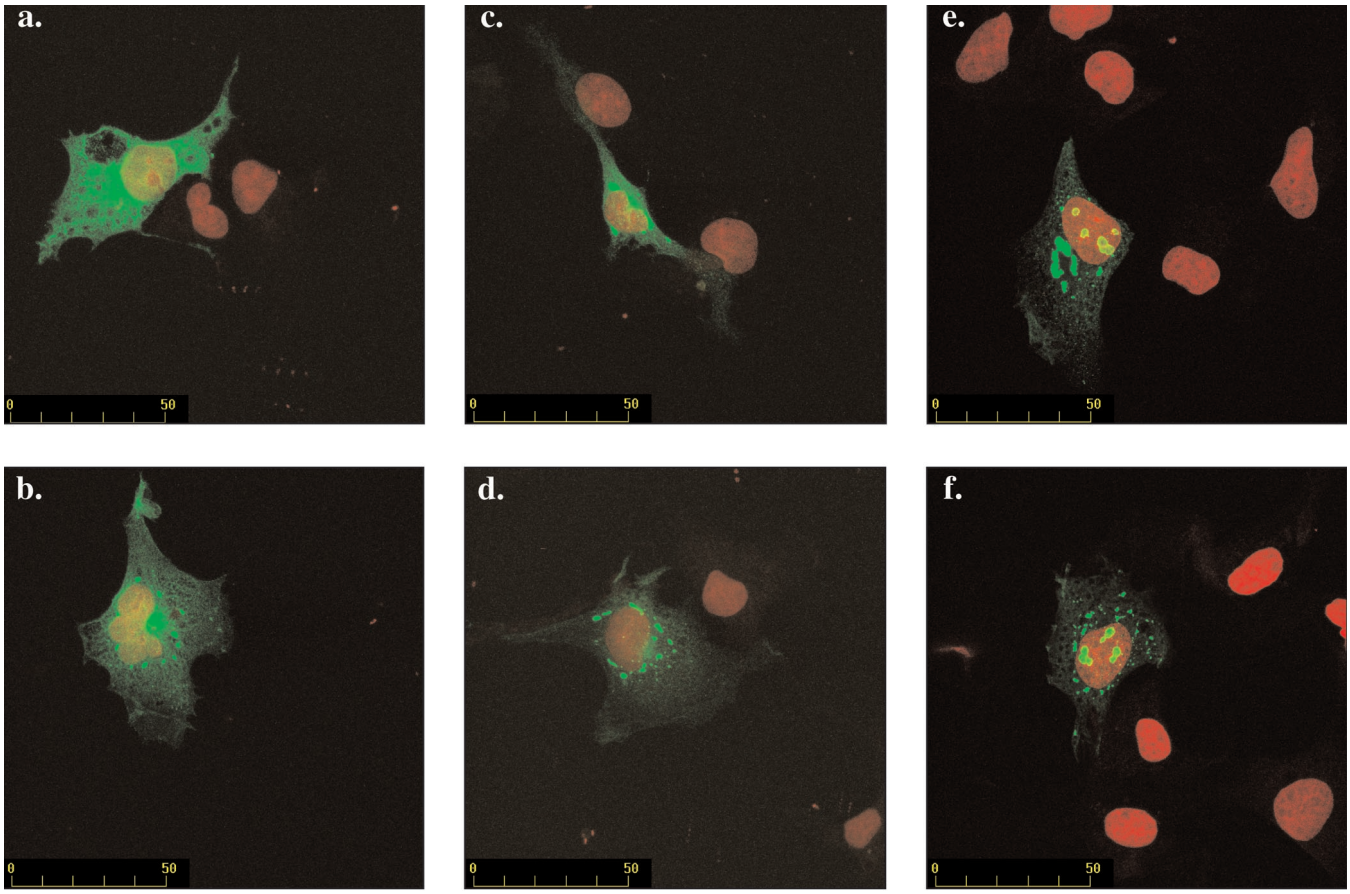

FIG. 5. **Confocal microscopy analysis of the subcellular localization of HIC p32 and p40 with an N- or C-terminal GFP tag *in vivo*.** COS7 cells were transfected with 20 μg each of expression vector encoding GFP-p32 (*a* and *b*), p32-GFP (*c* and *d*), GFP-p40 (*e*), or p40-GFP (*f*). Cells were cultivated on the glass slides and then analyzed by fluorescence 24 h after transfection. Analysis of the *green, red,* and *yellow* fluorescence was performed with confocal microscope. The *yellow* color results from the merging of the green fluorescence of GFP-tagged proteins and red staining (propidium iodide) of the cell nucleus.

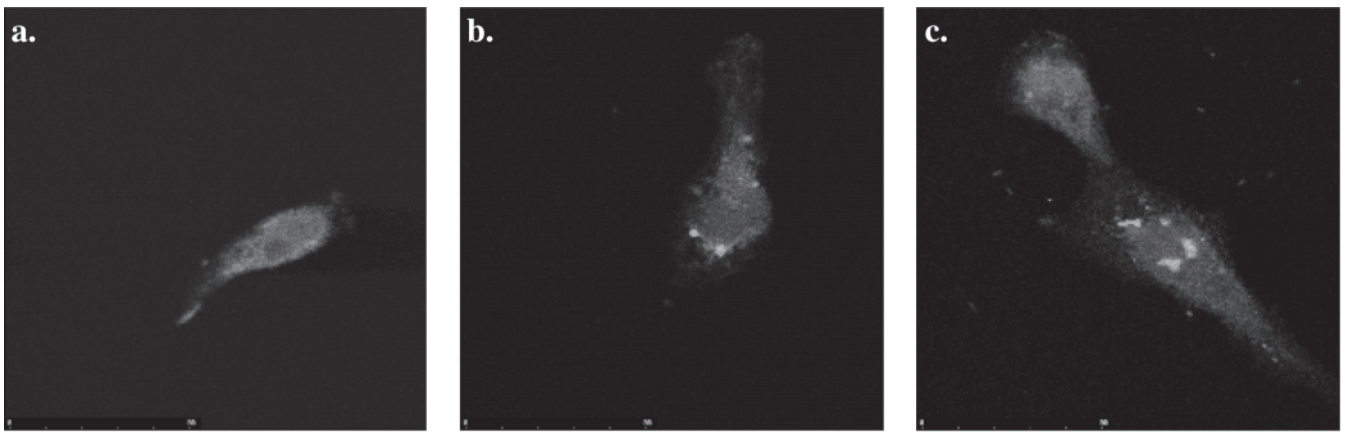

FIG. 6. **Confocal microscopy analysis of the subcellular localization of HIC p32 and p40 with a C-terminal *myc* tag *in vivo*.** COS7 cells were transfected with 20 μg of pcDNA3.1(−)/Myc-His expressing p32 (*a* and *b*) or p40 (*c*) fused to the *myc* epitope. Microscopy analysis was performed as described in the legend of Fig. 5.

p40 was not detected by both approaches suggesting that p40 is weakly expressed *in vivo*, probably because GTG is a poor translation initiator. To check the existence of p40 *in vivo*, the HIC coding sequence under the control of the wild type leader (from nucleotide 95 to 1328, see Fig. 2) was cloned into the vector pEGFP-N1, in frame with the GFP nucleotide sequence. If both HIC isoforms are expressed *in vivo*, the products synthesized from our construct should correspond to p40- and p32-GFP fusion proteins, easily detectable by fluorescence microscopy. Indeed, after transfection of COS7 cells, different patterns of staining were observed with a confocal microscope. At first, a diffuse cytoplasmic staining (Fig. 8*a*) was detected confirming that p32 is expressed *in vivo* from HIC cDNA. We also found a granular staining in the cytoplasm (Fig. 8, *a–c*), a pattern common to p32-GFP and p40-GFP (see Fig. 5, *d* and *f*). Finally, staining around and in the nucleoli (Fig. 8*c*), similar to the pattern described for p40-GFP (Fig. 5*f*), was observed. In conclusion, taken together, our analyses demonstrate that both p32 and p40 can be synthesized *in vivo*.

HIC p32 and p40 Show Opposite Effects on HTLV-I and

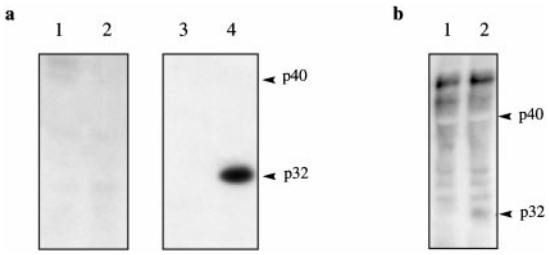

FIG. 7. **Detection of p32 *in vivo* by Western blot (*a*) or by immunoprecipitation (*b*).** *a*, 100 μg of total protein extracts were analyzed by SDS-PAGE and immunoblotting using control serum obtained from an uninjected rabbit (*lanes 1* and *2*) or anti-HIC serum (*lanes 3* and *4*). Protein extracts were prepared from 293T cells transfected with empty pcDNA3.1(−)/Myc-His (*lanes 1* and *3*) or with pcDNA3.1(−)/Myc-His containing the HIC coding sequence under the control of the wild type leader (*lanes 2* and *4*). *b*, HIC p32 was immunoprecipitated from MT-2 cell lysate (20×10^6 cells) using control serum (*lane 1*) or anti-HIC serum (*lane 2*) and protein A-Sepharose. Precipitates were analyzed by SDS-PAGE and immunoblotting with anti-HIC serum. The predicted positions of p40 and p32 are designated by *arrows* on the *right* of the immunoblots.

HIV-1 LTRs—Our results suggest that HIC protein belongs with I-mfa to the same family of proteins that is characterized by the presence of a specific cysteine-rich C-terminal domain. Therefore, by analogy with I-mfa, we postulated that HIC should be involved in gene expression regulation. Moreover, we first isolated the HIC cDNA clone from a cDNA library of MT-2, a T-cell line persistently infected by HTLV-I and producing high quantity of viral particles (47). For all these reasons and in order to try to understand the function of HIC, we investigated whether HIC was able to regulate the expression of HTLV-I LTR. For this purpose, transient cotransfection assays were carried out using a luciferase reporter gene driven by the HTLV-I promoter. The transfection assays were performed in CEM cells in the presence or absence of Tax. Although Tax alone was found to activate the expression of luciferase reporter gene by about 20-fold as expected, luciferase activity was over-stimulated in the presence of p32 and p40, by 50- and 60-fold, respectively (Fig. 9). This effect is comparable to the stimulations that have been described for cellular factors such as CREB, CREB-2, Ets1, and TBP known to cooperate with Tax for activating transcription from the HTLV-I LTR (20, 26, 29, 48).

By analogy with I-mfa, we postulated that the I-mfa-like domain of HIC could be involved in this regulation. To find out if this was indeed the case we constructed a mutated HIC p40 (HICΔ) deleted of its C-terminal domain. HICΔ has a removal of the last 101 amino acid residues encompassing the I-mfa-like domain (see Fig. 4). No over-stimulation of the luciferase gene expression from the HTLV-I LTR was detected with this mutant, although it was stably expressed in transfected cells (Fig. 10). This result confirms that the stimulation of luciferase expression from HTLV-I LTR by the wild type HIC, in the presence of Tax, is significant. Finally, to determine whether the stimulation was specific to HTLV-I, the effects of HIC were also tested on two other viral activators, the herpes simplex virus transactivator VP16 and the HIV-1 Tat protein. Whereas HIC has no effect on the VP16 activity (data not shown), the results obtained with Tat were unexpected. In the presence of HIC, the activation by Tat from HIV-1 LTR was down-regulated, especially with p40 (Fig. 11*a*). Such an effect was not detected in the presence of HICΔ (Fig. 11*b*). Taken together, our results suggest that HIC is able to modulate the expression of the HTLV-I and HIV-1 genomes, and its I-mfa-like domain is involved in this effect. To define better the function of this domain, we constructed a GAL4-HIC fusion protein in which the GAL4 DNA-binding domain was linked in frame to HIC (amino acids 120–355; see Fig. 4*a*). GAL4-HIC was assayed by using the luciferase reporter vector pG5luc, which contains five GAL4-binding sites upstream of a minimal TATA box. We found that GAL4-HIC was unable to stimulate the luciferase expression (Fig. 12*a*); this result suggests that HIC has no transcriptional activation domain. On the other hand, the GAL4-Tax and GAL4-Tat activities were stimulated 4.5- and 3-fold, respectively, in the presence of HIC (Fig. 12). Thus, HIC has the same activator effect on both Tax and Tat when these viral factors are positioned on the promoter.

DISCUSSION

In this report, we describe the cloning of a 4,152-bp full-length human cDNA encoding a novel protein designated HIC. Comparison of the nucleotide sequence of HIC cDNA with all the sequences of EMBL and GenBank™ data bases reveals significant homologies between the C-terminal amino acids of HIC protein and the specific C-terminal domain of I-mfa, a cellular factor known to inhibit the transcriptional activity of *MyoD* family members and Mash2 (38, 39). By *in vitro* translation, we demonstrated that the HIC cDNA directed the expression of two different proteins, p32 and p40. These two protein isoforms are synthesized from two initiation sites of translation present in the corresponding mRNA. Site-directed mutagenesis indicates that p32 is generated from the ATG codon at position 591 and that p40 is an N-terminal extension of p32 produced by alternative translation initiation at an upstream GTG codon. Although ATG is essentially used as initiation codon for eukaryotic mRNAs, there are several examples of cellular mRNAs where non-ATG codons located upstream of an ATG codon are also used as initiator for the synthesis of protein isoforms. For instance, these include proto-oncogenes such as c-*myc*, *int-2*, and *pim-1* (49–51), as well as the genes encoding the fibroblast growth factors (52, 53), the Wilms' tumor suppressor (54), and the high affinity neurotensin receptor (55). However, in human cells, whereas TTG and CTG have often been found as initiation codons, GTG has rarely been described as initiator (56–58). Non-ATG start codons can be used to create a functionally distinct form of the same protein. Sometimes, extended proteins show intracellular localizations different from their shorter counterparts (50, 59). For HIC protein, p32 is mainly distributed throughout the cytoplasm, whereas p40 is targeted to the nucleus. This observation can be explained by the presence in HIC p40 of an N-terminal extension containing two clusters of basic amino acids that could direct nuclear import (60).

Despite their different intracellular localization, p32 and p40 have similar effects on the regulation of HTLV-I and HIV-1 expression. Indeed, we found that both HIC protein isoforms were able to stimulate the expression of a luciferase reporter gene driven by the HTLV-I LTR promoter in the presence of Tax but down-regulated expression from HIV-1 LTR promoter in the presence of Tat. However, the molecular mechanism involved in this regulation remains unclear. The effect of HIC on Tax is obviously indirect since HIC is unable to interact directly with Tax (data not shown). Besides, we have observed that I-mfa is also capable of stimulating the HTLV-I transcription in the presence of Tax,[2] suggesting that the C-terminal cysteine-rich domain, common to both proteins, is involved in this regulation. Abolishment of HIC activity by deleting its I-mfa-like domain confirms this hypothesis. The I-mfa-specific cysteine-rich domain is required for binding to some bHLH factors (38). This interaction masks the nuclear localization signal of the transcriptional factor and thus retains the bHLH in the cytoplasm. It is unlikely that such a similar mechanism is involved in retroviral promoter regulation by HIC in T-cells. The bHLH proteins have not been described as regulators of

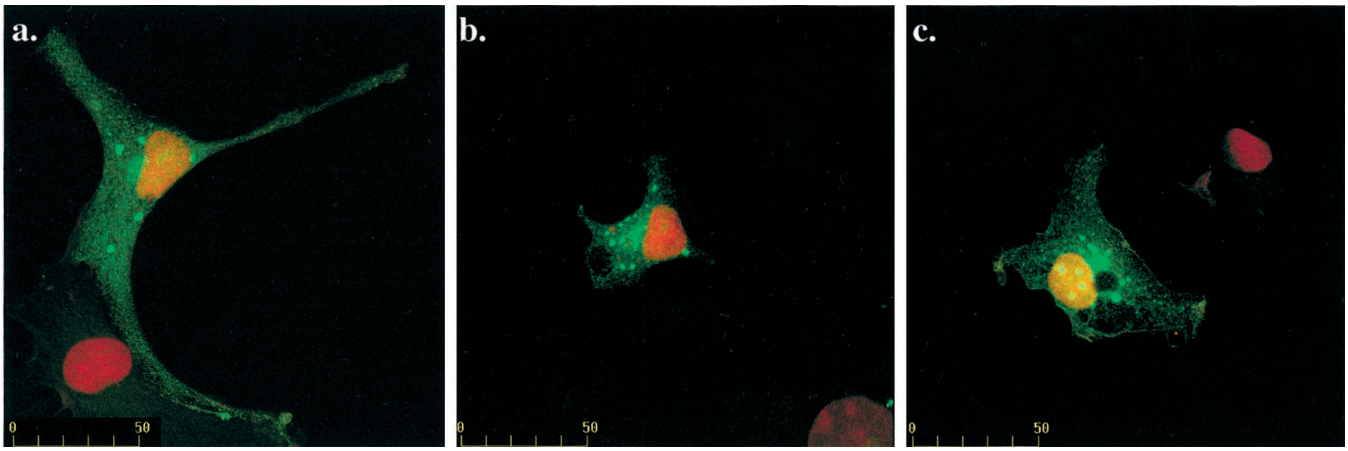

FIG. 8. **Analysis of HIC expression *in vivo* from the wild type HIC cDNA by confocal microscopy.** COS7 cells were transfected with pEGFP-N1 containing GFP nucleotide sequence in frame with the HIC coding sequence under the control of the wild type leader. Analysis of transfected cells was carried out as described in the legend of the Fig. 5. The three different patterns, which have been observed, are shown in *a–c*.

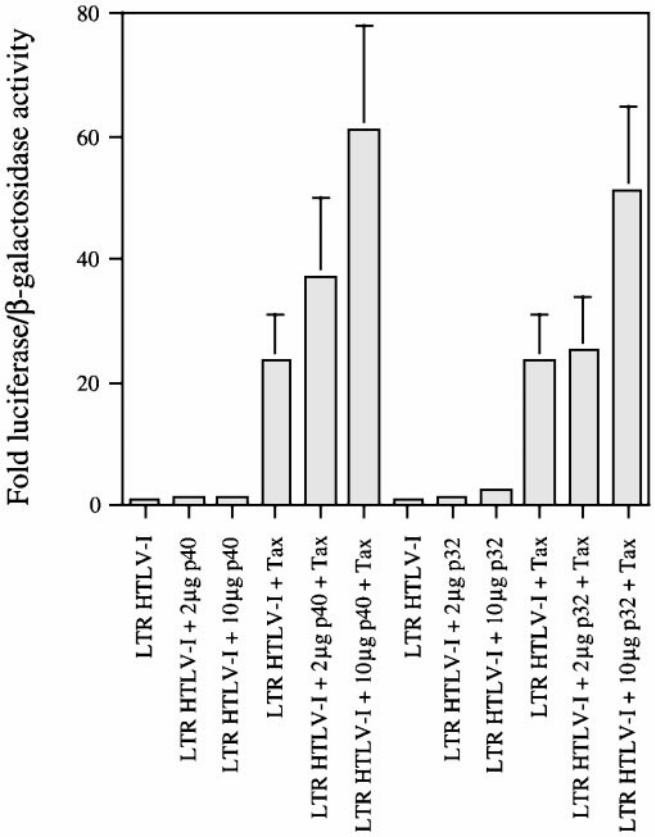

FIG. 9. **Stimulation of HTLV-I LTR by HIC p32 and p40.** CEM cells were cotransfected with 2 μg of HTLV-I LTR-luciferase, 5 μg of pACβ1 (β-galactosidase-containing reference plasmid), 1 μg of Tax expression vector pSG-Tax or empty pSG-5 vector, and 0, 2, or 10 μg of pcDNA3.1/His expressing p32 or p40. Luciferase values were normalized for β-galactosidase activity and are expressed as fold increase relative to that of cells transfected with 2 μg of HTLV-I LTR-luciferase, 5 μg of pACβ1, 1 μg of pSG-5, and 10 μg of empty pcDNA3.1/His. Values are the means ± S.D. (n = 3).

the HIV-1 and HTLV-I transcription. On the other hand, Tax is known to repress the activity of bHLH factors (61–63), probably through competition for common transcriptional regulators as described recently for another transcriptional factor, c-Myb. Tax antagonizes the c-Myb transcriptional activity by competing for CBP (64). In our model system, HIC protein could stimulate the transcription of the HTLV-I LTR promoter by preventing the interaction of bHLH proteins with transcriptional regulators such as CBP which, in turn, would bind to Tax. This hypothesis could also explain why HIC protein is able to stimulate Tat activity when this viral factor is positioned on the HIV-1 LTR promoter since Tat is also known to recruit transcriptional regulators to the viral promoter (65–67). On the other hand, the down-regulating effect of HIC on Tat unbound to the viral promoter still remains unclear. A direct interaction between HIC and Tat could be involved in such a negative effect on Tat especially since both proteins localize to the nucleolus (68). Experiments are under way to evaluate further this possibility.

Another intriguing question is the putative involvement of CD4 in the control of HIC activity. Indeed, we have previously reported that monoclonal antibodies directed against the Ig CDR3-like region in the extracellular domain 1 of CD4 can inhibit HIV-1 transcription in the MT-2 T-cell line superinfected by this virus, by inducing signals that repress NF-κB activation (43, 69). We have established that the transcription of the *lck* gene, which encodes a cytoplasmic CD4-associated kinase linking CD4 to signal transduction cascade, is repressed in MT-2 cells (43, 69). This result suggests that inhibition of HIV-1 transcription in these cells is a consequence of signal transduction probably mediated by an unidentified factor capable of associating with CD4. It is noteworthy that several reports demonstrate that some CD4 signals do not require Lck thereby suggesting the involvement of unknown molecules (70–72). In trying to identify this factor by the yeast two-hybrid approach by using the CD4 cytoplasmic tail as a bait, we isolated two cDNA clones encoding the C-terminal domain of HIC. We have also found by two-hybrid assay that HIC binds more efficiently to the wild type CD4 cytoplasmic tail than to a mutant form with cysteine mutations in its cytoplasmic fragment, suggesting that cysteines of HIC and CD4 are likely involved in the protein-protein interaction.[3] Interestingly, HIC is capable of down-regulating HIV-1 transcription. However, confocal microscopy analyses do not argue in favor of CD4/HIC cellular colocalization. Moreover, we also failed to coimmunoprecipitate HIC and CD4 from cotransfected cells. For these reasons, we cannot presently claim that the interaction between HIC and CD4 really occurs in MT-2 cells. Yet, we can expect that further characterization of exact mechanisms involved in the regulation of HTLV-I and HIV-1 LTR expression by HIC may contribute to answer this question.

Our results suggest that p32 is the main isoform of HIC protein in HTLV-I-infected T-cells. p32 presents many similar-

[3] N. Coudronnière and J.-M. Mesnard, unpublished results.

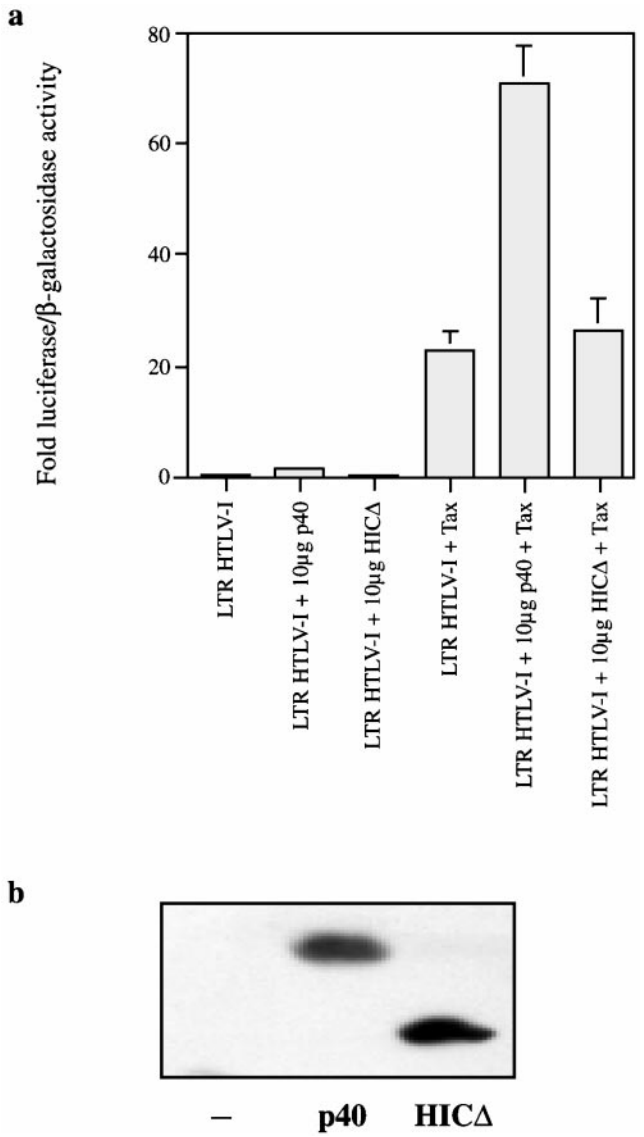

FIG. 10. **The I-mfa-like domain of HIC is involved in the stimulation of the HTLV-I LTR expression.** *a*, CEM cells were cotransfected with 2 μg of HTLV-I LTR-luciferase, 5 μg of pACβ1, 1 μg of pSG-Tax or empty pSG-5, and 10 μg of empty pcDNA3.1/His or 10 μg of pcDNA3.1/His expressing HIC p40 or HICΔ. Values are the means ± S.D. (n = 3). *b*, expression of the wild type and mutated HIC proteins in cotransfected CEM cells was checked by Western blotting by using the anti-Xpress serum: −, cells cotransfected with empty pcDNA3.1/His; *p40,* cells cotransfected with pcDNA3.1/His expressing HIC p40; *HIC*Δ*,* cells cotransfected with pcDNA3.1/His expressing HICΔ.

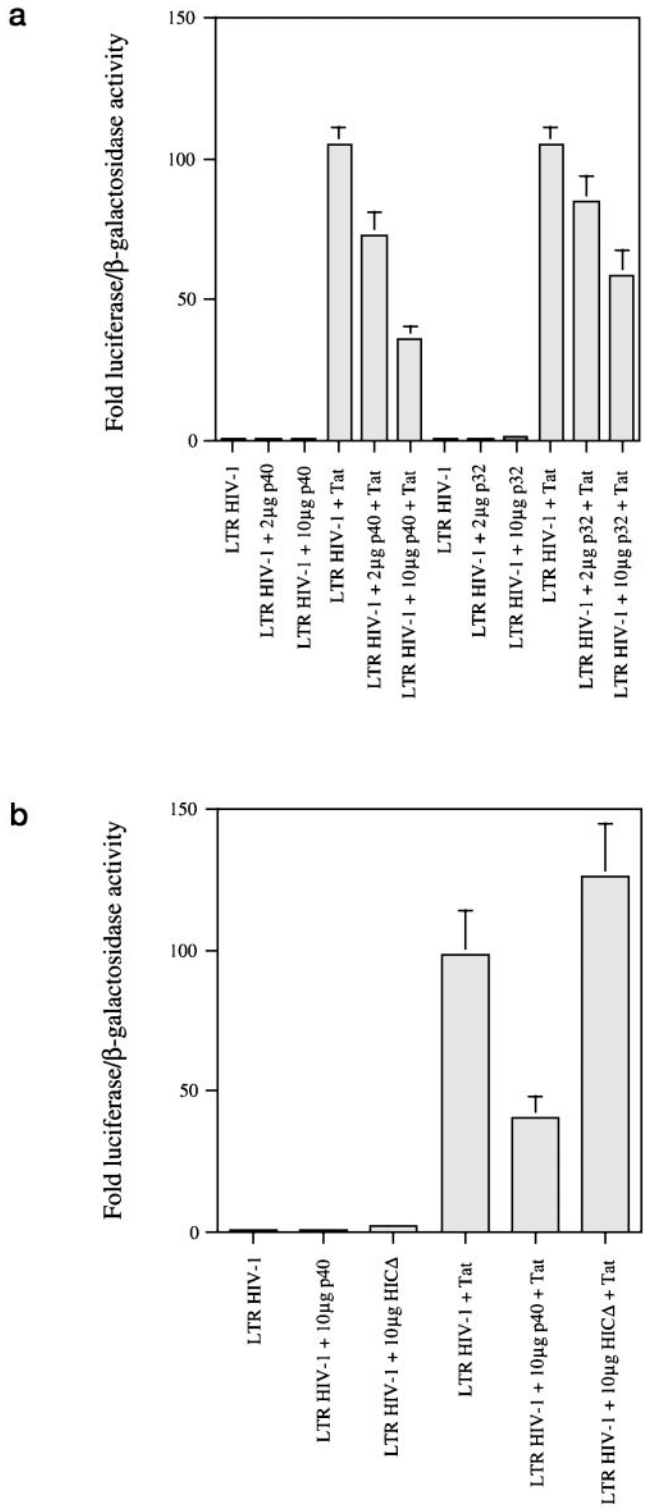

FIG. 11. **Down-regulation of HIV-1 LTR by HIC p32 and p40.** To test the effects of HIC p32 and p40 (*a*) and HICΔ (*b*) on the HIV-1 LTR-luciferase, CEM cells were transfected as described in the legends of Fig. 9 and 10, but with HIV-1 LTR and Tat expression vector instead of HTLV-I LTR and pSG-Tax. Values are the means ± S.D. (n = 3).

ities with I-mfa as follows: (i) their translation is initiated at an ATG codon, (ii) their C-terminal region shares 77% identical amino acids, (iii) p32 and I-mfa have the same size, 246 amino acids in length, (iv) both p32 and I-mfa are mainly located in the cytoplasm, (v) p32 and I-mfa are involved in gene expression regulation, and (vi) their C-terminal domain is indispensable to this function. On the other hand, p40 seems to be weakly expressed in HTLV-I-infected T-cells. However, several observations suggest that p40 could also play a major role *in vivo*. At first, the 5′ structure of HIC mRNA is completely different from that of I-mfa mRNA. Whereas the ATG initiation codon of I-mfa cDNA is located at position 150, the ATG initiator of p32 is located at position 591 of HIC cDNA. Such a very long leader is rare for eukaryotic mRNAs and suggests that the regulation of HIC protein expression *in vivo* is more complex than that of I-mfa. Second, preliminary data[2] indicate that the N-terminal domain of p40 is involved in the import of HIC in the nucleus, showing that this domain has an essential function *in vivo*. Finally, p40 is more efficient than p32 in regulating the expression of a luciferase gene driven by the HTLV-I or HIV-1 LTR.

In conclusion, we propose that HIC and I-mfa represent two members of a new family of gene expression regulators, char-

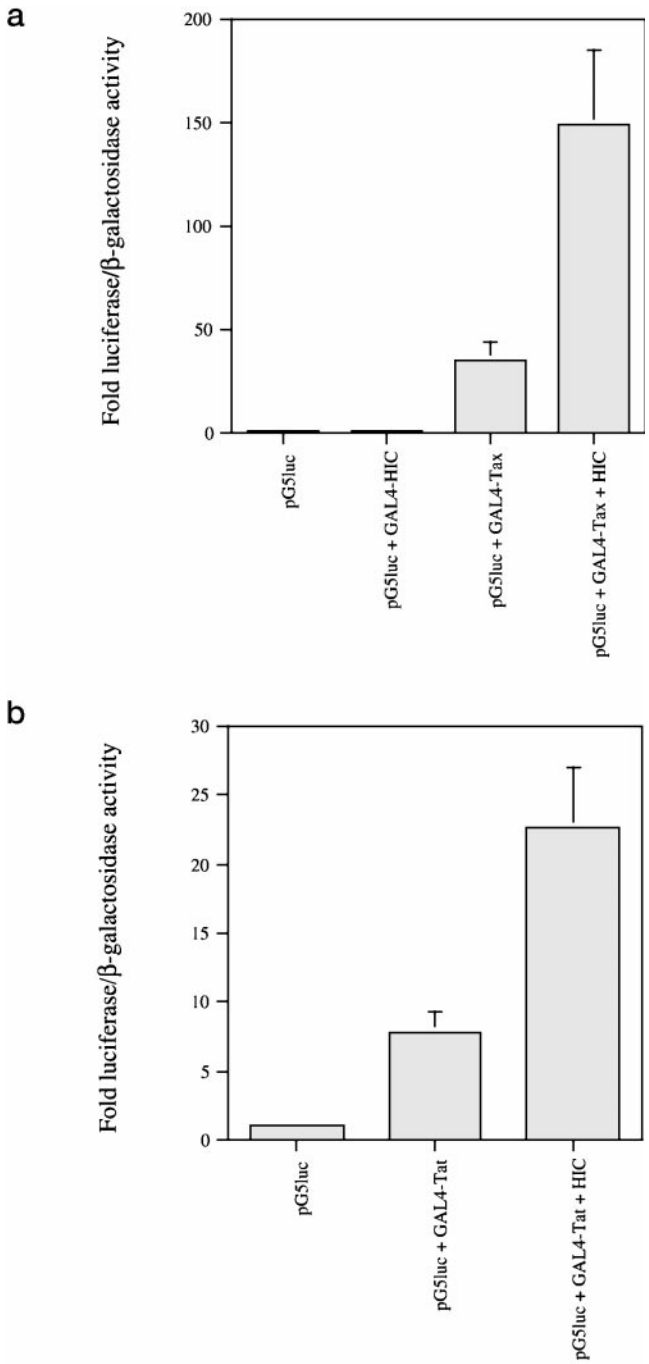

FIG. 12. **Stimulation of GAL4-Tax and GAL4-Tat activities by HIC.** *a*, CEM cells were cotransfected with 2 μg of the luciferase reporter vector pG5luc, 5 μg of pACβ1, and 2 μg of a eukaryotic vector expressing a GAL4-HIC fusion protein. The GAL4-Tax activity was also tested with pG5luc in the presence of 10 μg of empty pcDNA3.1/His or 10 μg of pcDNA3.1/His expressing HIC p40. Luciferase values were normalized for β-galactosidase activity and are expressed as fold increase relative to that of cells transfected with 2 μg of pG5luc, 5 μg of pACβ1, and 12 μg of empty pcDNA3.1/His. Values are the means ± S.D. (n = 3). *b*, the same experiments were also performed with GAL4-Tat.

acterized by a particular cysteine-rich C-terminal domain. Our study opens a new perspective in understanding the role of these new factors in the pathology of diseases such as AIDS and T-cell leukemia.

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