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Human Cytomegalovirus mtrII Oncoprotein Binds to p53 and Down-Regulates p53-Activated Transcription†

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The 79-amino-acid (79-aa) open reading frame (UL111a) gene within morphological transforming region II (mtrII) of human cytomegalovirus strain Towne has been shown to transform rodent cells in vitro (J. Thompson, J. Doniger, and L. J. Rosenthal, Arch. Virol. 136:161–172, 1994). Moreover, a translation termination linker (TTL) mutant of mtrII that coded for the first 49 aa of mtrII oncoprotein (designated TTL_{49}) was sufficient for malignant transformation, whereas a TTL mutant that coded for the first 24 aa (designated TTL_{24}) was not. The current study demonstrates the binding of mtrII oncoprotein to the tumor suppressor protein p53 both in vivo using transiently transfected cells and in vitro using labeled proteins. Furthermore, the C-terminally truncated mtrII protein TTL_{49}, but not truncated protein TTL_{24}, bound to p53. The mtrII binding domain mapped to the N-terminal region of p53, residues 1 to 106, with a critical region from aa 27 to 44, whereas the p53 binding domain of mtrII protein was the first 49 aa. Furthermore, mtrII inhibited p53-activated transcription, indicating its ability to alter p53-directed cellular regulatory mechanisms. mtrII oncoprotein was detected both in stably transfected NIH 3T3 cell lines and human cytomegalovirus-infected HEL 299 cells (as early as 12 h after infection) in the perinuclear region and in the nucleus. mtrII-transformed cell lines, at both early and late passage, exhibited high levels of p53 with a 15-fold-extended half-life. However, p53-activated transcription was suppressed in these cells in spite of the increased p53 levels. Finally, the results with wild-type mtrII and its TTL mutants with respect to p53 binding, p53-activated transcription, and transforming ability suggest that the mechanism of mtrII transformation is linked to both p53 binding and disruption of p53 cell regulation.

Human cytomegalovirus (HCMV), a member of the herpesvirus family, is an important human pathogen that causes widespread primary and recurrent infections ranging from mild asymptomatic to severe congenital infections. HCMV infections result in a variety of life-threatening diseases, particularly in transplant recipients, patients with AIDS, and other immunocompromised individuals (49). It has also been implicated in coronary restenosis after angioplasty (57). Moreover, HCMV has been identified by both serological and molecular studies in several human cancers, including cervical carcinoma, adenocarcinoma of the prostate and colon, and Kaposi's sarcoma (49, 72), suggesting a possible etiologic role.

No reports have as yet established a direct link between HCMV genes and human cancer. However, the oncogenic potential of both infectious and UV-irradiated HCMV was originally established by in vitro studies (1, 49). Moreover, HCMV infection induced a rapid and substantial increase in the transcription of the cellular oncogenes *jun*, *fos*, and *myc* (4, 5). Other investigations have led to the identification of three morphological transforming regions (mtr) (Fig. 1). mtrI, a 558-bp noncoding fragment located on an *Xba*I-*Hin*dIII fragment of strain AD169, transformed cells by a transcriptional enhancer mechanism (16, 39). mtrII and mtrIII, located within the Towne *Xba*I-E DNA fragment, were reported and characterized by our laboratory (7, 11, 20–22, 45, 60). In contrast to mtrI and mtrIII, only mtrII sequences were retained and expressed in transformed and tumor-derived cell lines (11), suggesting their role in maintaining the transformed phenotype.

Results demonstrating that the 79-amino-acid (79-aa) open reading frame (ORF) (59), also designated UL111A, was the mtrII transforming gene were recently obtained. Insertion of a translation termination linker (TTL) into codon 24 of the 79-aa ORF resulted in inhibition of its transforming activity and provided evidence that mtrII gene expression was required for transforming ability. Moreover, the translation of the first 49 aa was sufficient for transforming activity. As such, the HCMV 79-aa ORF has been designated the mtrII oncogene.

PCR analyses have demonstrated the presence of HCMV mtrII oncogene sequences in 36% of cervical biopsies, while none of the tissues were positive for other HCMV sequences (64). Sequences amplified from nonmalignant tissues of congenitally HCMV-infected infants contained deletions within the 79-aa ORF (30). The occurrence of intact mtrII sequences in malignant tissues has suggested that HCMV infection could serve as a cofactor responsible for progression of human papillomavirus-associated dysplasia to malignancy. Understanding mechanisms of mtrII transformation may help elucidate the putative role of HCMV in human malignancy. Studies of other DNA tumor virus oncogenes such as simian virus 40 (SV40) T, adenovirus E1A and E1B, and human papillomavirus (HPV) type 16 and 18 E6 and E7 have shown that their viral oncoproteins bind to cellular tumor suppressor protein p53 and/or Rb (10, 51, 53, 67, 68). These interactions resulted in the

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† L. J. Rosenthal dedicates this paper to J. M. Biastock and E. R. Picard, who both died from adenocarcinoma of the lung.

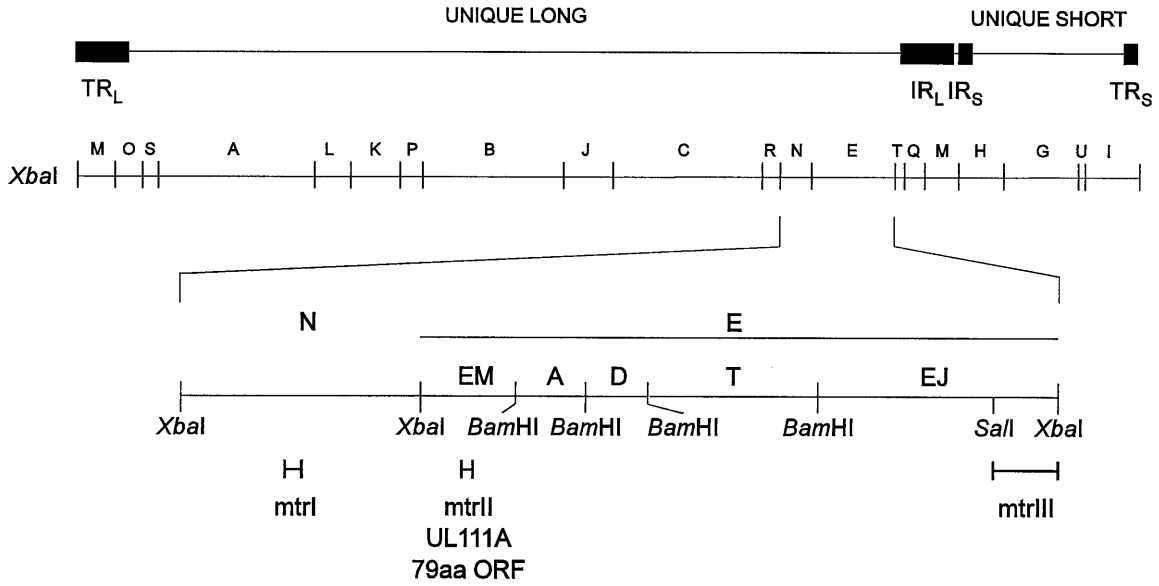


FIG. 1. Restriction map of HCMV Towne strain showing the location of the mtrII oncogene. mtrI has been identified in strain AD169. IR_L and IR_S, inverted long and short repeats, respectively.

inactivation of required checkpoints in the cell cycle, giving rise to uncontrolled cellular DNA replication and transformation.

In the present study, the mtrII oncoprotein was detected in HCMV-infected cells and in both transiently and stably transfected cells. By immunofluorescence and immunohistochemical staining assays, mtrII was localized predominantly in the perinuclear region and in the nucleus. The mtrII oncoprotein bound to p53 in coimmunoprecipitations in vitro and in vivo. The p53 binding domain within mtrII was within the first 49 aa, while the N-terminal region of p53 (residues 1 to 106) was mapped as the mtrII binding domain. Additional studies indicated that the mtrII oncoprotein expressed in transiently transfected human Jurkat T cells had the ability to inhibit p53-activated transcription. This suggested the potential of mtrII to affect cell cycle regulatory mechanisms. Moreover, as a result of a 15-fold extension in the p53 half-life, the level of p53 was elevated in stably mtrII-transformed NIH 3T3 cell lines. In contrast, p53-activated transcription was suppressed in these cell lines. Thus, the ability of the HCMV mtrII oncoprotein to bind to p53 and inactivate its transactivation function suggests a putative mechanism for mtrII oncogene transformation.

MATERIALS AND METHODS

Plasmids. Plasmids pCHC79orf, pCHC79orf-TTL_{49}, and pCHC79orf-TTL_{24}, expressing the mtrII oncoprotein and the corresponding truncated peptides in a mammalian expression system, described previously (59), are designated pCHC/mtrII, pCHC/mtrII-TTL_{49}, and pCHC/mtrII-TTL_{24}, respectively. pET17b/mtrII (provided by D. Palmer), containing the mtrII 79-aa ORF cloned into the pET17b expression vector (Novagen), was used for in vitro expression of the mtrII oncoprotein by use of the TNT system (Promega) and for obtaining purified mtrII oncoprotein from bacterial cells. Plasmids pBK/mtrII and pBK/mtrII(A) were derived by cloning the pCHC/mtrII *Xba*I subfragment containing the mtrII 79-aa ORF into the vector pBK-CMV (Stratagene) in either the sense or the antisense orientation, respectively. Plasmid pCMV/p53, expressing wild-type human p53 from the HCMV immediate-early promoter, and plasmid p53G5BCAT (reporter plasmid with a chloramphenicol acetyltransferase [CAT] gene driven by a promoter containing two copies of the p53-responsive element from the ribosomal gene cluster) were gifts from S. J. Kim (National Cancer Institute, Bethesda, Md.). pT7Hup53, containing human p53 cloned in a pT7 vector, and its deletion derivatives 3M, 3R, 4U, 4D, Δ11, and Δ18 were gifts from D. Lane (35, 40). Plasmids pU3RCAT and pCMV-tax (18) and human immunodeficiency virus (HIV) LTR-CAT and pCMV-tat (13) have been described previously.

Cells, virus infection, and transfection. NIH 3T3 cells and WI38 cells (fibroblasts that overexpress a mutant form of p53) were grown in Dulbecco's modified Eagle's medium (Quality Biologicals) supplemented with 2 mM glutamine, 100 U each of penicillin and streptomycin per ml, and 10% fetal bovine serum for WI38 cells or 7% bovine calf serum for NIH 3T3 cells. Human Jurkat T cells were grown in RPMI 1640 (Quality Biologicals) supplemented with 10% fetal bovine serum, 2 mM glutamine, and penicillin-streptomycin (100 U/ml each).

HEL 299 cells (American Type Culture Collection) were grown in Eagle's minimal essential medium (Cellgro) supplemented with 10% fetal bovine serum, 2 mM glutamine, and penicillin-streptomycin (100 U/ml each). HCMV strain Towne stock was prepared by infecting confluent monolayers of HEL 299 cells at a multiplicity of infection of 0.001 PFU per cell as described previously (44). Cells were seeded at a split ratio of 1:3 in 150-cm-diameter petri dishes and infected 24 h later with HCMV at a multiplicity of infection of 10 PFU per cell. Infected cells were harvested at designated times postinfection, washed twice with phosphate-buffered saline (PBS), scraped into 10 ml of PBS, collected by centrifugation, and frozen at −70°C.

Transient transfections of Jurkat cells were carried out in a Bethesda Research Laboratories electroporation unit as previously described (24). For stable transfection, 4 μg of the plasmid vector pBK-CMV, pBK/mtrII, or pBK/mtrII(A) were linearized by *Hpa*I digestion and transfected into 2×10^5 NIH 3T3 cells in 60-mm-diameter petri dishes by using Lipofectamine (GIBCO BRL). Cells were selected 48 h posttransfection with 500 μg of geneticin (Sigma) per ml. Selected colonies were isolated and maintained in the presence of 100 μg of geneticin per ml. Cell line BK-1 was derived from plasmid pBK-CMV; BK/mtrII-1 and BK/mtrII-2 were two independently derived clones from plasmid pBK/mtrII, and BK/mtrII(A)-1 was derived from plasmid pBK/mtrII(A).

Antibodies. A rabbit polyclonal antibody, Ab-471, provided by C. Hall and G. Jay (American Red Cross), was raised against the C-terminal 20-mer peptide of the mtrII oncoprotein. The antiserum was immunoglobulin G (IgG) purified with an Avid Chrom protein A antibody purification kit (Sigma Immunochemicals). Purified antibody (25 μl) was used for immunoprecipitation and for Western blot (immunoblot) analysis (1:200 dilution). Mouse monoclonal antibody Ab-1 (PAb-421) (Oncogene Sciences) is an IgG2a isotype that reacts against both human wild-type and mutant p53. Immunoprecipitation reactions employed 1.0 μg of Ab-1, while 10 μg was used for Western blot analysis. Mouse monoclonal antibody Ab-2 (PAb 1801) (Oncogene Sciences) is an IgG1 isotype that reacts with the N terminus of human p53. Immunoprecipitation reactions employed 1.0 μg of Ab-2. 12CA5, a mouse monoclonal antibody against hemagglutinin (HA) and Tab 169-172, a mouse anti-Tat antibody, were used as nonrelevant controls in immunoprecipitation assays.

Immunoprecipitation and Western blot analysis. Extracts of transfected or infected cells were prepared by a gentle lysis procedure in TNE buffer (100 mM Tris [pH 7.5], 150 mM NaCl, 10 mM EDTA) with 0.1% Nonidet P-40 (NP-40) for 30 min on ice.

Coimmunoprecipitation studies were performed with [^{35}S]methionine-labeled proteins synthesized by using the TNT transcription and translation coupled system (Promega) according to the manufacturer's protocols. Equal counts per minute of proteins translated from plasmid pET17b (vector control) or pET17b/

mtrII plus pT7Hup53 or its deletion derivatives 3M, 3R, 4U, 4D, Δ11, and Δ18 were added to binding buffer (50 mM NaCl, 50 mM Tris [pH 8.0], and 0.5% NP-40).

To synthesize C-terminally truncated mtrII proteins, the pET17b/mtrII plasmid was digested prior to TNT synthesis with either *Bgl*II to obtain a transcribed-translated runoff product, designated TTL_{49}, corresponding to the first 49 aa of mtrII, or with *Nae*I to obtain a product designated TTL_{24}, containing the first 24 aa of mtrII.

In vitro-synthesized proteins or cell extracts were added to primary antibody and mixed by rotation for 1 h at 4°C. Protein A-Sepharose beads (25 μl of a 50% solution) (Pharmacia) were added, and the solution was mixed for 30 min. The beads were then collected by centrifugation, washed three times in 100 mM NaCl–100 mM Tris (pH 8.0)–1% NP-40, and resuspended in 25 μl of Laemmli buffer. The beads were mixed in a vortex, collected by centrifugation, and heated at 95°C for 5 min to elute the proteins, which were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In vitro-synthesized proteins were detected by autoradiography and/or phosphorimager analysis. Proteins extracted from transfected or infected cells were transferred to polyvinylidene difluoride Immobilon-P membranes (Millipore) in a Mini-Transblot electrophoretic transfer cell (Bio-Rad) and detected by Western blotting. Binding of the secondary antibody was detected with an enhanced chemiluminescence kit (Amersham).

To quantitate the percentage of input mtrII that coimmunoprecipitated with p53, an aliquot of input [^{35}S]methionine-labeled mtrII was analyzed by SDS-PAGE concurrently with the coimmunoprecipitations. The levels of mtrII in the input and coimmunoprecipitated lanes were measured by phosphorimager analysis using Image-Quant software.

Estimation of p53 half-life. The half-life of p53 in cells was determined by cycloheximide-Western blot assays (32). Cultures were treated with 5 μg of cycloheximide per ml, and proteins were extracted at appropriate times with a gentle lysis buffer (described below) containing 10 mM dithiothreitol. Equal amounts of total protein extracted at each time point were separated by SDS-PAGE and analyzed by Western blotting using anti-p53 antibody Ab-1. The level of p53 protein detected in each lane of the Western blot autoradiograph was measured with an LKB laser densitometer.

Immunofluorescence and immunohistochemistry. Cells grown as monolayers on glass slides (Labtek) were fixed in situ with 4% paraformaldehyde for 10 min at room temperature, while cells grown in tissue culture dishes were trypsinized, transferred to polylysine-coated slides, allowed to dry at 37°C for 30 min, and fixed in acetone for 5 min at −20°C. Fixed cells were blocked with 0.1% bovine serum albumin at 37°C for 1 h, washed with PBS for 5 min, and incubated with primary anti-mtrII antibody Ab-471 (1:10 dilution) at 37°C in a humidified chamber for either 1 h (acetone fixed) or overnight (paraformaldehyde fixed). Fluorescein isothiocyanate (FITC)-conjugated secondary antibody was used for detection of mtrII oncoprotein. Mounting medium [50% glycerol and 5% DAPI (4,6-diamidino-2 phenyl-indole)-antifade (*p*-phenylenediamine) solution in PBS] (20 μl) was added, a coverslip was placed on top and sealed, and the cells were observed with a Zeiss Axiophot fluorescent microscope. For immunohistochemistry, noninfected and HCMV-infected HEL 299 cells were trypsinized and fixed in acetone as described above. mtrII oncoprotein was stained with Ab-471 which was detected with anti-rabbit IgG conjugated to horseradish peroxidase as described elsewhere (2).

CAT assays. Cell extracts of transiently transfected cells were prepared by three cycles of freeze-thawing followed by centrifugation to remove cell debris. Equal amounts of total protein, determined with a Bio-Rad protein assay kit, were used in all assays. CAT activity was assayed in the presence of 0.125 μCi of [^{14}C]chloramphenicol (Amersham) and 5 mM acetyl coenzyme A (Sigma) at 37°C for 1 h. Acetylated and nonacetylated forms of chloramphenicol were separated by thin-layer chromatography and quantitated by phosphorimager analysis.

RESULTS

mtrII oncoprotein is expressed in pBK/mtrII-transfected and HCMV-infected cells and localized predominantly to the perinuclear region. Proteins extracted from Jurkat cells transiently transfected with pBK/mtrII were immunoprecipitated with anti-mtrII antibody (Ab-471), separated by SDS-PAGE, and Western blotted with Ab-471. mtrII oncoprotein (Fig. 2A, lane 3) migrated as a 12-kDa protein (just below the 14-kDa marker) and had a lower mobility than bacterially expressed mtrII oncoprotein (lane 2), indicating that it may be posttranslationally modified in mammalian cells. No corresponding band was observed in nontransfected Jurkat cells (lane 1) or when preimmune rabbit serum was used for immunoprecipitation (data not shown).

Geneticin-selected NIH 3T3 clonal lines BK/mtrII-1 and -2, BK/mtrII(A)-1, and BK-1 were established by transfection with

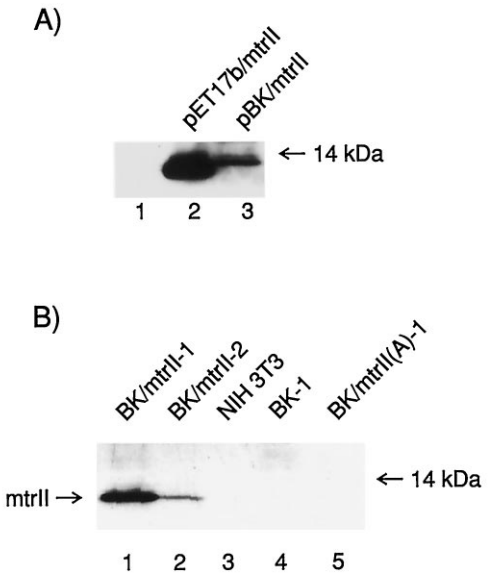


FIG. 2. Expression of mtrII oncoprotein in transiently and stably transfected cells. Cells were washed in PBS, gently lysed in a lysis buffer with 0.1% NP-40, and immunoprecipitated with Ab-471 (raised against the C-terminal 20-mer peptide of the mtrII oncoprotein). Proteins were separated by SDS-PAGE. mtrII oncoprotein was detected with Ab-471 followed by horseradish peroxidase-conjugated anti-rabbit IgG antibody and detected by enhanced chemiluminescence. The 14-kDa molecular mass marker is shown. (A) Lane 1, nontransfected Jurkat cells; lane 2, bacterially expressed mtrII oncoprotein that was not immunoprecipitated; lane 3, pBK/mtrII-transfected Jurkat cells. (B) Stably transfected NIH 3T3 cell lines. Cell lines BK-1, BK/mtrII-1 and -2, and BK/mtrII(A)-1 were derived by transfection with pBK-CMV, pBK/mtrII, and pBK/mtrII(A), respectively.

pBK/mtrII, pBK/mtrII(A), or the vector pBK-CMV, respectively, and examined for expression of mtrII oncoprotein (Fig. 2B). mtrII oncoprotein was observed only in BK/mtrII-1 and -2 cells (Fig. 2B, lanes 1 and 2). The level of mtrII expression was consistently higher in BK/mtrII-1 cells in repeated experiments.

mtrII cell lines were next examined by immunofluorescence for the location of mtrII oncoprotein. After acetone fixation, the cells were treated with both DAPI, to identify the nucleus (Fig. 3A), and anti-mtrII antibody to identify mtrII protein (Fig. 3B). FITC staining of mtrII oncoprotein was observed predominantly in the perinuclear region as well as in the nucleus in both BK/mtrII-1 and -2 cells (Fig. 3B1 and B2) but not in BK/mtrII(A)-1 cells (Fig. 3B3) or in NIH 3T3 or BK-1 cells (data not shown). When cells were fixed with paraformaldehyde, which preserves the cell structure better than acetone fixation, staining was also predominantly perinuclear (Fig. 3C1 and C2). Again, no mtrII staining was observed in the antisense BK/mtrII(A)-1 cells (Fig. 3C3). The detection of mtrII oncoprotein by immunohistochemistry with anti-mtrII antibody in HCMV-infected HEL 299 cells as early as 24 h (Fig. 3D1), but not in noninfected cells (Fig. 3D2), indicated its expression during HCMV replication. Moreover, mtrII protein was detected in infected cells at 12 to 72 h (data not shown).

The mtrII oncoprotein bound to p53. Binding of the mtrII oncoprotein to p53 was examined by coimmunoprecipitation in vitro (Fig. 4A). ^{35}S-labeled mtrII and p53 proteins were synthesized by coupled transcription-translation reactions in vitro, mixed, and immunoprecipitated with anti-p53 antibody Ab-1 (which recognizes an epitope in the C terminus) or Ab-2 (which recognizes an epitope in the N terminus). Coimmunoprecipitated mtrII protein was observed when either Ab-1 or

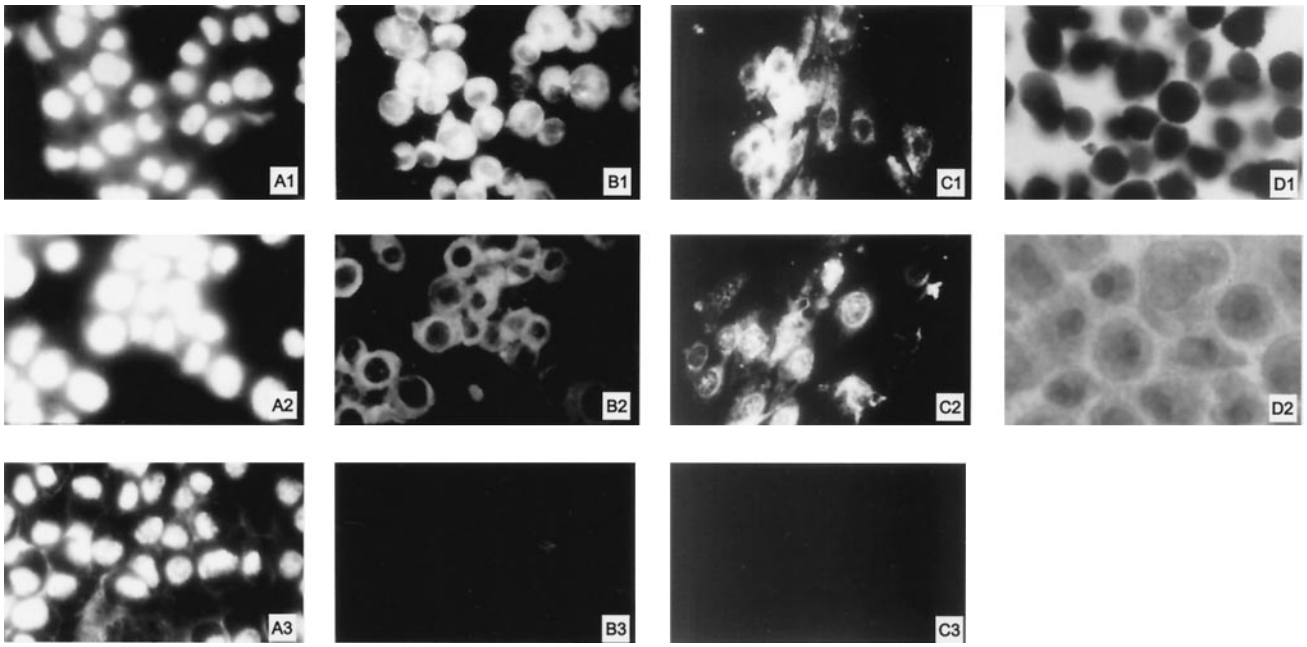


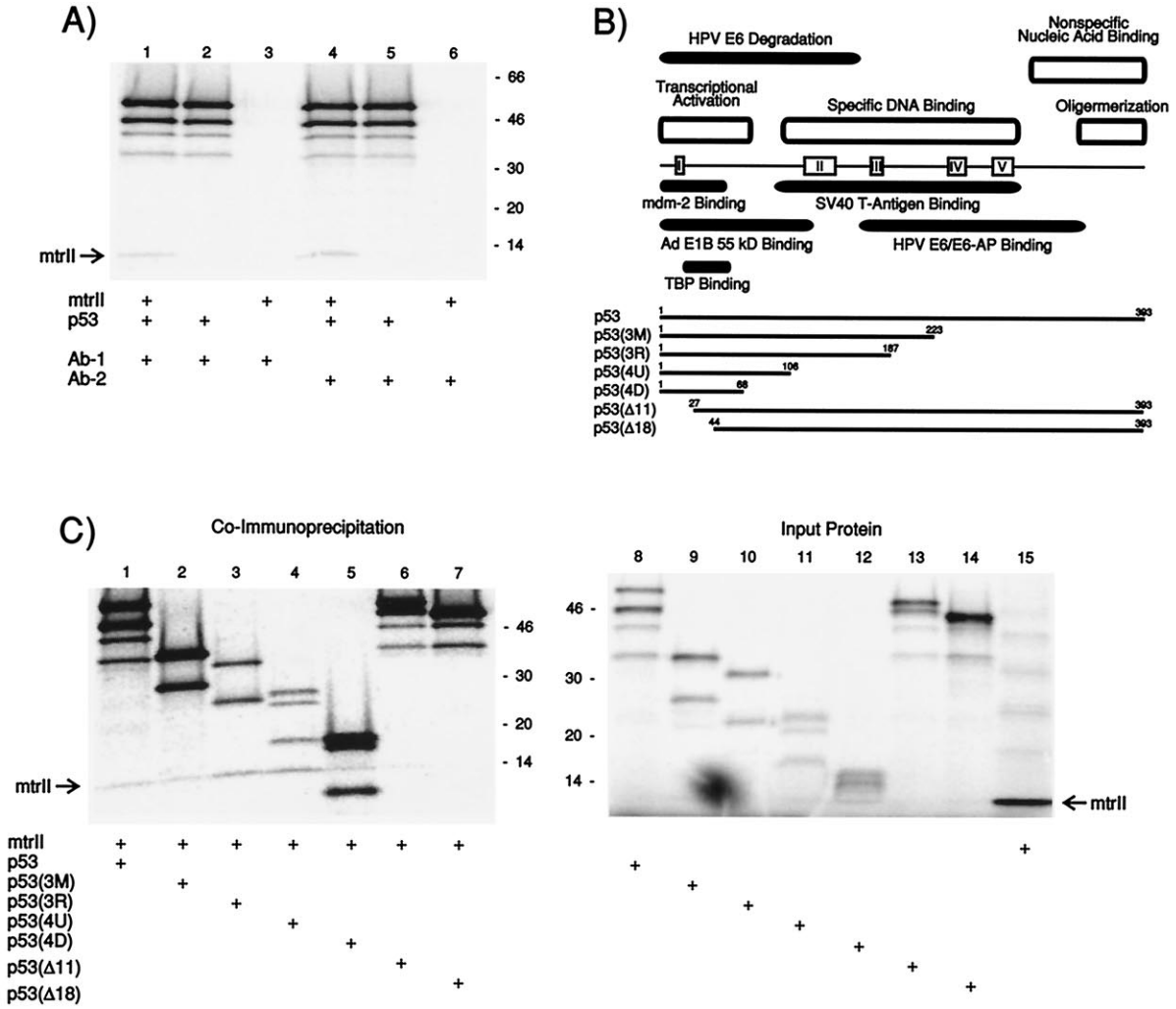
FIG. 3. MtrII oncoprotein localized predominantly to the perinuclear region. Stably transfected NIH 3T3 cell lines, BK/mtrII-1 (A1 and B1), BK/mtrII-2 (A2 and B2), and BK/mtrII(A)-1 (A3 and B3), were fixed in acetone and costained with DAPI to locate the nucleus and anti-mtrII antibody Ab-471 to identify mtrII protein. The results of DAPI fluorescence (A1 to A3) and of anti-mtrII fluorescence (detected by anti-rabbit IgG coupled to FITC) (B1 to B3) are shown. A similar series of cells were grown on slides, fixed with paraformaldehyde, and stained with Ab-471 antibody (C1 to C3). HEL 299 cells infected with HCMV for 24 h (D1) and noninfected cells (D2) were stained with Ab-471 and detected by anti-rabbit IgG coupled to horseradish peroxidase.

Ab-2 was used (Fig. 4A, lanes 1 and 4). However, no mtrII protein was immunoprecipitated when mixed with either anti-p53 antibody in the absence of p53 (Fig. 4A, lanes 3 and 6). Furthermore, no protein was found at the position of mtrII when either anti-p53 antibody was mixed with p53 in the absence of mtrII (Fig. 4A, lanes 2 and 5). The data demonstrated that mtrII protein bound to p53.

mtrII protein was examined for binding to truncated p53 proteins to locate the region within p53 required for binding to mtrII. Both C- and N-terminally truncated p53 proteins were ^{35}S labeled in vitro. mtrII oncoprotein coimmunoprecipitated with truncated p53 proteins 3M, 3R, 4U, and 4D (Fig. 4C). Reduced binding was observed with Δ11, while no binding was observed with Δ18, which lacked the first 44 aa at the N terminus of p53. Input proteins are presented to show the positions of full-length and truncated p53 proteins produced during in vitro synthesis. The low-molecular-weight band sometimes observed in input p53 protein preparations at the position of mtrII protein (Fig. 4C, lanes 8 to 14) was never found when full-length p53 (Fig. 4A, lanes 2 and 5) or truncated p53 (data not shown) in the absence of mtrII was immunoprecipitated with the appropriate anti-p53 antibody. Furthermore, no pET17b vector transcription-translation products were immunoprecipitated in the presence of p53 by either anti-p53 antibody (data not shown). The percentage of the input mtrII oncoprotein that coprecipitated with wild-type p53 and each of the p53 truncated proteins was calculated (Fig. 4D). About 12% of input mtrII oncoprotein consistently coprecipitated with wild-type p53. About 18% was bound to 4U (residues 1 to 106), with intermediate amounts with 3M and 3R. Little or no binding occurred with Δ11 and Δ18. The results demonstrated that the primary mtrII oncoprotein binding domain of p53 was localized between aa 1 and 106, with a critical region located between residues 27 and 44.

In vitro coimmunoprecipitation analysis was also employed to determine the region within mtrII oncoprotein required for binding to p53 protein. To synthesize [^{35}S]methionine-labeled C-terminally truncated mtrII oncoproteins, the plasmid pET17b/mtrII was digested with *Bgl*II or *Nae*I to yield products containing residues 1 to 49 and 1 to 24, respectively, after runoff transcription-translation. These corresponded to mtrII TTL mutants used previously to study transforming ability (59). Wild-type mtrII oncoprotein and the TTL_{49} protein product coimmunoprecipitated with p53, whereas the TTL_{24} protein product did not (Fig. 5A, lanes 1 to 3). The faint band detected at the position of full-length mtrII in the TTL_{24} lane

FIG. 4. mtrII oncoprotein bound to the N-terminal domain of p53. Proteins were synthesized by in vitro transcription and translation in the presence (or absence) of [^{35}S]methionine with the Promega TNT kit. (A) Coimmunoprecipitation of mtrII with wild-type p53 using anti-p53 C-terminus antibody Ab-1 (lanes 1 to 3) and anti-p53 N-terminus antibody Ab-2 (lanes 4 to 6). Equal counts per minute of labeled proteins as indicated were mixed with the indicated antibody and incubated for binding. Immunoprecipitated proteins were analyzed by SDS-PAGE. Positions of molecular mass markers (in kilodaltons) are shown on the right. (B) Map of human wild-type and truncated p53 proteins. The locations of the DNA binding domains (66), transcriptional activation and TBP binding domains (29), an oligomerization domain (63), conserved regions (56), E6/E6-AP binding and degradation domains (34), mdm-2 and adenovirus (Ad) E1B (55-kDa protein) binding domains (28), and the SV40 T-antigen binding domain (50) are shown. (C) Coimmunoprecipitation of mtrII oncoprotein with wild-type or truncated p53. Equal counts per minute of labeled proteins as indicated were mixed and incubated for binding. Each mixture was immunoprecipitated with anti-p53 antibody (Ab-2 for p53, Δ11, and Δ18 and Ab-1 for the other truncated p53 proteins) and analyzed by SDS-PAGE. Positions of molecular mass markers (in kilodaltons) are shown. All lanes (1 to 7) with p53 wild-type and truncated proteins contain degradation products. The highest p53 band in each lane is the full-length product. (D) Percentages of input mtrII oncoprotein that were coimmunoprecipitated with wild-type or truncated p53.


A)
1 2 3 4 5 6
- 66
- 46
- 30
- 20
- 14
mtrII →
mtrII + + + + +
p53 + + + + +
Ab-1 + + +
Ab-2 + + +
B)
HPV E6 Degradation
Nonspecific Nucleic Acid Binding
Transcriptional Activation
Specific DNA Binding
Oligermerization
I II III IV V
mdm-2 Binding
SV40 T-Antigen Binding
Ad E1B 55 kD Binding
HPV E6/E6-AP Binding
TBP Binding
p53 1 393
p53(3M) 1 223
p53(3R) 1 187
p53(4U) 1 106
p53(4D) 1 66
p53(Δ11) 27 393
p53(Δ18) 44 393
C)
Co-Immunoprecipitation
1 2 3 4 5 6 7
- 46
- 30
- 20
- 14
mtrII →
mtrII + + + + + + +
p53 +
p53(3M) +
p53(3R) +
p53(4U) +
p53(4D) +
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8 9 10 11 12 13 14 15
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Immunoprecipitated Protein

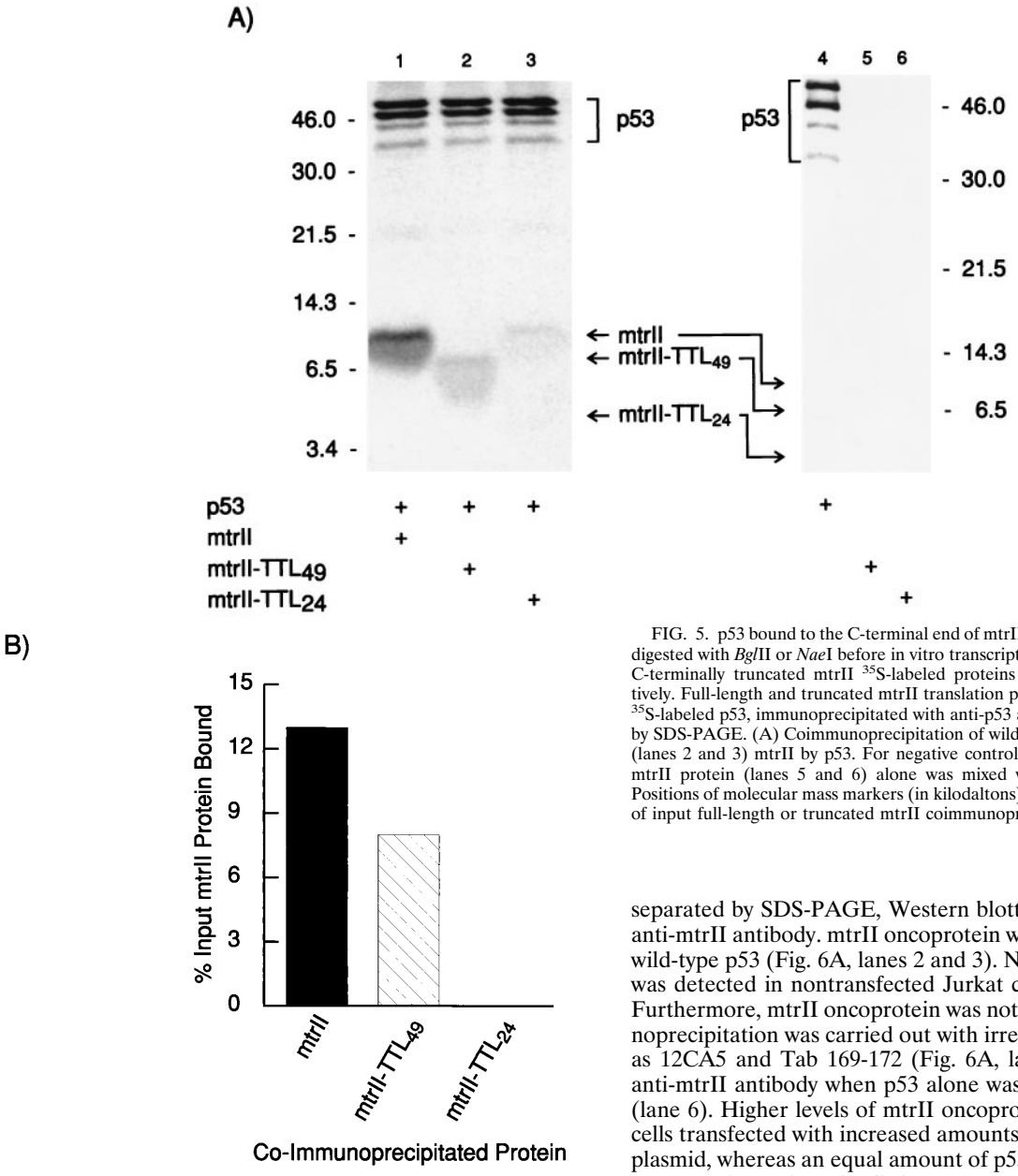

FIG. 5. p53 bound to the C-terminal end of mtrII. Plasmid pET17b/mtrII was digested with *Bgl*II or *Nae*I before in vitro transcription-translation to synthesize C-terminally truncated mtrII ^{35}S-labeled proteins TTL_{49} and TTL_{24}, respectively. Full-length and truncated mtrII translation products were incubated with ^{35}S-labeled p53, immunoprecipitated with anti-p53 antibody Ab-1, and analyzed by SDS-PAGE. (A) Coimmunoprecipitation of wild-type (lane 1) and truncated (lanes 2 and 3) mtrII by p53. For negative controls, p53 (lane 4) or truncated mtrII protein (lanes 5 and 6) alone was mixed with Ab-1 (lanes 5 and 6). Positions of molecular mass markers (in kilodaltons) are shown. (B) Percentages of input full-length or truncated mtrII coimmunoprecipitated by p53.

arose from the low level of full-length mtrII protein translated from undigested pET17b/mtrII that was observed after analysis of input protein. The TTL_{49} and TTL_{24} protein products alone do not react with the p53 antibody Ab-1 (Fig. 5A, lanes 5 and 6). Thus, the mtrII oncoprotein binding domain for p53 was within aa 1 to 49, with a critical region between aa 25 and 49. Moreover, mtrII-TTL_{49} protein did not bind p53 as efficiently as the wild-type mtrII oncoprotein (Fig. 5B), indicating that aa 50 to 79 are necessary for complete binding.

mtrII oncoprotein bound to p53 in vivo and down-regulated p53-activated transcription. The binding of mtrII oncoprotein to p53 was next studied in vivo. Jurkat cells (with the p53 gene deleted) were coelectroporated with pBK/mtrII (5 or 10 μg) and pCMV/p53 (10 μg). Cell extracts were prepared at 24 h postelectroporation and immunoprecipitated with anti-p53 antibody Ab-1 or control antibodies 12CA5 (anti-HA) and Tab 169-172 (anti-Tat). The immunoprecipitated complexes were separated by SDS-PAGE, Western blotted, and detected with anti-mtrII antibody. mtrII oncoprotein was coprecipitated with wild-type p53 (Fig. 6A, lanes 2 and 3). No corresponding band was detected in nontransfected Jurkat cells (Fig. 6A, lane 1). Furthermore, mtrII oncoprotein was not detected when immunoprecipitation was carried out with irrelevant antibodies such as 12CA5 and Tab 169-172 (Fig. 6A, lanes 4 and 5) or with anti-mtrII antibody when p53 alone was transfected into cells (lane 6). Higher levels of mtrII oncoprotein were observed in cells transfected with increased amounts (10 μg) of pBK/mtrII plasmid, whereas an equal amount of p53 was detected in both extracts of transfected cells by Western blot analysis (data not shown).

The effect of the mtrII oncogene on p53-activated transcription was studied by employing a reporter CAT construct, p53G5BCAT, with an upstream p53-responsive element. CAT activity from p53G5BCAT (2 μg) was increased >70-fold when Jurkat cells were cotransfected with 0.2 μg of pCMV/p53. Cotransfection with equal amounts of pCMV/p53 and pCHC/mtrII decreased transactivation by 40% (Fig. 6B). The inhibition of p53-activated transcription reached approximately 85% with a 25-fold excess of pCHC/mtrII. Moreover, no inhibition of basal p53G5BCAT transcription (transfection without pCMV/p53) by mtrII was found (data not shown). Although little or no inhibition was observed with pCHC/mtrII-TTL_{49} at 2.0 μg, a similar inhibition was seen with 5.0 μg. Moreover, partial inhibition was observed with pCHC/mtrII-TTL_{24} at both concentrations (Fig. 6B). mtrII inhibition of p53-activated transcription was specific, because mtrII failed to inhibit human T-cell leukemia virus type 1 (HTLV-1) *tax*-

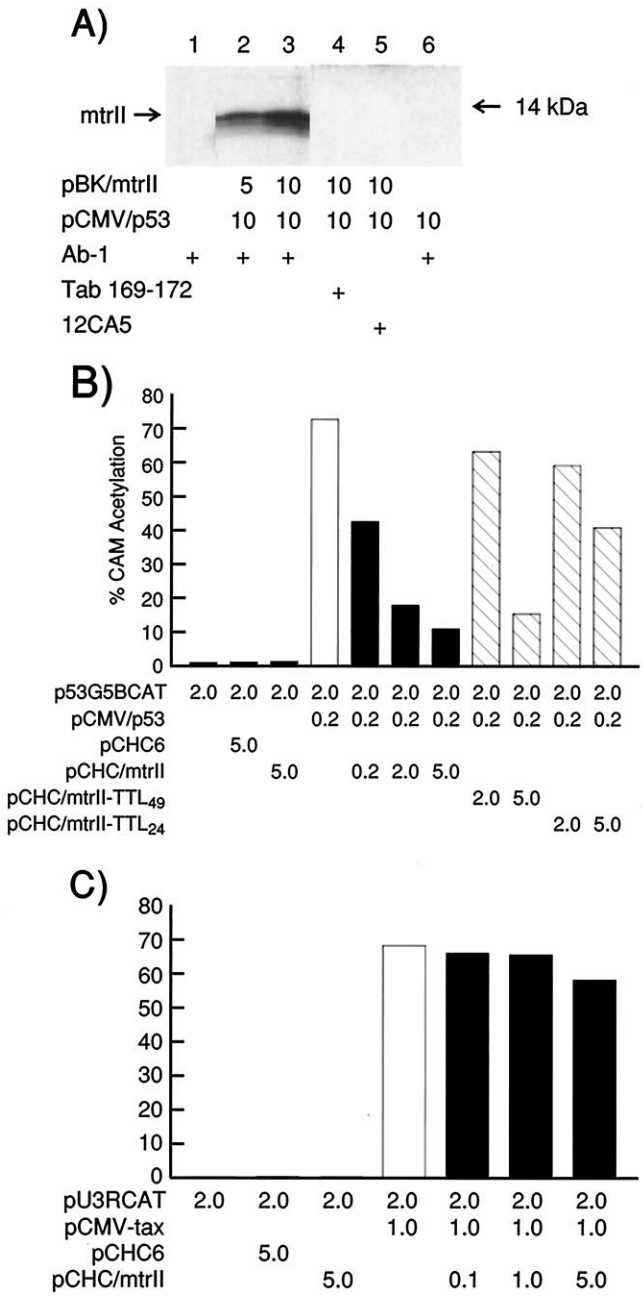

FIG. 6. mtrII oncoprotein bound to p53 in vivo and inhibited p53-activated transcription. (A) Coimmunoprecipitation of mtrII oncoprotein with p53 in transiently transfected Jurkat cells. Cells were coelectroporated with 10 μg of pCMV/p53 (lanes 2 to 6) and 0 (lane 6), 5 (lane 2), or 10 (lanes 3 to 5) μg of pBK/mtrII. At 24 h, cell extracts were prepared, immunoprecipitated with anti-p53 antibody Ab-1 (lanes 1, 2, 3, and 6) or control nonrelevant antibodies 12CA5 (anti-HA, lane 5) and Tab 169-172 (anti-Tat, lane 4), separated by SDS-PAGE, and Western blotted with Ab-471 antibody. The position of the 14-kDa molecular mass marker is indicated. (B) Inhibition of p53-activated transcription by mtrII. Jurkat cells were coelectroporated with 2 μg of p53G5BCAT, a p53-responsive reporter CAT construct, plus the indicated amounts (in micrograms) of pCMV/p53, pCHC6, pCHC/mtrII, pCHC/mtrII-TTL_{49}, and/or pCHC/mtrII-TTL_{24} DNA. At 24 h posttransfection, cell extracts were prepared and assayed for CAT activity. Results for cotransfections with no mtrII, wild-type mtrII, and TTL mutant mtrII are shown (open, solid, and striped bars, respectively). CAM, chloramphenicol. (C) Jurkat cells were coelectroporated with 2 μg of the reporter CAT plasmid, pU3RCAT, containing the HTLV-1 long terminal repeat promoter, plus the indicated amounts (in micrograms) of pCMV-tax, pCHC6, and/or pCHC/mtrII DNA and analyzed as described above.

directed transactivation of pU3RCAT (Fig. 6C) or Tat-driven HIV LTR-CAT (data not shown).

p53 protein was stabilized but functionally suppressed in NIH 3T3 cells expressing the mtrII oncoprotein. Early- and later-passage cells expressing mtrII oncoprotein were examined for the levels of p53 by Western blot analysis using the anti-p53 antibody Ab-1 (Fig. 7A and B). In nontransfected NIH 3T3 cells, p53 was barely detectable because of its relatively short half-life of 6 to 20 min (Fig. 7 and reference 42, 46, and 48). Levels of p53 were 10- to 20-fold higher at both early (2.5 weeks; Fig. 7A) and later (11 weeks; Fig. 7B) passages in BK/mtrII-1 and -2 cells than those in NIH 3T3, BK-1, and BK/mtrII(A)-1 cells. Moreover, p53 levels were comparable to those in human fibroblast WI38 cells used as a positive control.

mtrII-expressing cells were examined to measure the extent of p53-activated transcription. BK/mtrII-2 and BK-1 (vector control) cell lines were transfected with the reporter CAT construct p53G5BCAT (2 or 4 μg). In each cell line, the percentage of CAT conversion was greater with 4 μg of the reporter plasmid. Interestingly, p53-activated CAT activity in BK/mtrII-2 was reduced compared with that in BK-1 cells (Fig. 7C). This indicated that in BK/mtrII-2 cells, less functional p53 was available to activate p53G5BCAT even though the detectable level of p53 was considerably higher.

High levels of p53 in mtrII-expressing cells were due to increased p53 half-life. The level of p53 was 10-fold higher in BK/mtrII-2 than in NIH 3T3 cells. The half-life of p53 in these cells was measured by cycloheximide-Western blot analysis to determine if p53 was more stable in mtrII-expressing cells. In BK/mtrII-2 cells, the half-life was 263 min, whereas it was 17 min in NIH 3T3 cells (Fig. 8), as expected (42, 46, 48). Thus, the high levels of p53 in mtrII-expressing cells resulted from the 15-fold-increased half-life.

DISCUSSION

Although members of the herpesvirus family have been associated with human cancers, no specific link between herpesvirus transforming genes and cellular tumor suppressor proteins has been established. In contrast, DNA viruses such as SV40, adenovirus, hepatitis B virus (HBV), and HPV induce transformation in rodent cells by expressing oncoproteins that bind to nuclear phosphoprotein Rb and/or p53, e.g., SV40 T antigen to both p53 and Rb (53), adenovirus E1A and HPV E7 to Rb (10, 68), and adenovirus type 12 E1B 55-kDa protein, HBV X protein, and HPV E6 to p53 (51, 65, 67). The mechanisms by which these protein-protein interactions inactivate p53 differ. The SV40 T antigen and adenovirus type 5 E1B 55-kDa protein both can sequester p53 into oligomeric complexes resulting in increased p53 stability and inhibition of p53 function (36, 51). HPV E6, on the other hand, induced degradation of p53 in a ubiquitin-dependent manner in vitro (15, 36, 52). Moreover, the HBV X protein bound to p53 and inhibited p53's sequence-specific DNA binding, transcriptional activity, and association with the transcription factor ERCC3, which is involved in nucleotide excision repair (65).

The 79-aa ORF sequence (UL111a), i.e., the mtrII oncogene, cloned into a mammalian expression vector caused focal as well as tumorigenic transformation of rodent cells. Moreover, the translation of the first 49 aa was sufficient for this transforming activity (59). The mtrII oncoprotein in transformed cells was localized predominantly to the perinuclear region (Fig. 3), consistent with the hydrophobicity of the N-terminal third of mtrII oncoprotein, indicative of a membrane binding domain (59).

mtrII oncoprotein bound to p53 in transiently transfected

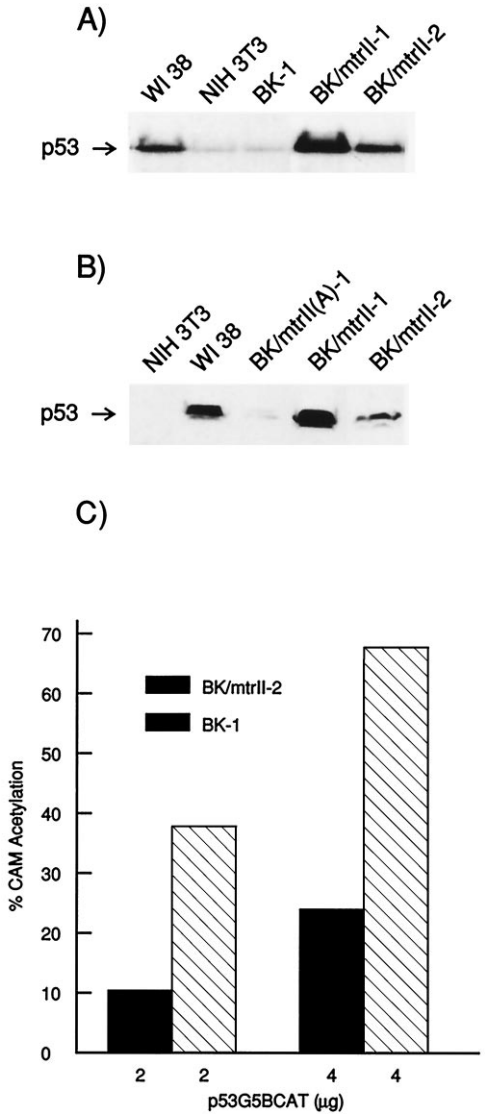

FIG. 7. Reduced p53-activated transcription in NIH 3T3 cell lines expressing mtrII oncoprotein and high levels of p53. (A and B) p53 levels in mtrII-expressing cells at early passage (2.5 weeks postselection) and later passage (11 weeks), respectively. Cell extracts were separated by SDS-PAGE and Western blotted for p53 with Ab-1 anti-p53 antibody. (C) p53-activated transcription in mtrII-expressing cells. BK/mtrII-2 and BK-1 cells were transfected with the indicated amounts of the p53-responsive CAT construct p53G5BCAT. Cell extracts were prepared 24 h posttransfection and assayed for CAT activity.

cells (Fig. 6). Furthermore, mtrII inhibited p53-activated transcription. Although the mechanism by which the latter occurs is not understood, binding is thought to be involved. mtrII-transformed cells, at both early and later passages, exhibited 10- to 20-fold-higher levels of p53 accompanied by a 15-fold-extended half-life (Fig. 7 and 8). Of interest, p53-activated transcription was suppressed in mtrII-expressing cells in spite of the increased p53 levels. Thus, the binding of mtrII oncoprotein to p53 and the resulting inhibition of its function(s) may explain the oncogenic potential of HCMV mtrII. Preliminary coimmunoprecipitation and glutathione *S*-transferase pull-down assays indicate that mtrII also binds to Rb protein (data not shown). Follow-up experiments are in progress to analyze the nature of this interaction and its biologic relevance.

In normal cells, wild-type p53 protein has a half-life of 6 to 20 min (42, 46, 48), whereas human tumor cells with mutations in the conserved domain of p53 (56) exhibit high p53 levels associated with a 10-fold or greater increase in half-life (14). The stabilization of p53 levels has also been observed in cell lines transformed by SV40 (27, 51), adenovirus (51), and HTLV-1, in which p53 remained wild type. The HBV X protein stabilized p53 by binding to it and restricting the movement of p53 into the nucleus (62). However, the mechanism by which mtrII oncoprotein stabilized p53 levels is unknown.

The mtrII oncoprotein binding domain of p53 was located in its N-terminal region (Fig. 4). mtrII oncoprotein coimmunoprecipitated with wild-type p53 and the truncated p53 (4U) containing aa 1 to 106. No binding and minimal binding were observed with p53 N-terminal truncations Δ18 and Δ11, which lacked the first 44 and 27 aa, respectively. This indicated that the minimal domain for efficient binding was between aa 1 and 106 and that aa 27 to 44 were critical.

The N-terminal region of p53 has several other properties. It contains the transcriptional activation domain as well as specific binding regions for TATA-binding protein (TBP) (29, 43, 61), adenovirus type 5 E1B (55 kDa) (23), HPV type 16 E6 (34), and the cellular protein mdm-2 (28), which regulates the levels of p53. It has been postulated that the N-terminal domain of p53 may mediate enhanced rates of transcription by interacting with the transcription machinery of the cell, i.e., TBP and/or other components of TFIID (29, 61). mdm-2 binds to the first 52 residues of p53 (41), and several hydrophobic amino acids in this region have been shown to be essential for this interaction. Binding of mdm-2 to p53 may sterically block interactions of p53 with the general transcription factors (41), although there is a report that mdm-2-bound p53 cannot bind to the p53-responsive element, suggesting that other mechanisms may also be involved. E1B (55 kDa) of adenovirus type 5 can inhibit p53 function by binding to it and sequestering the p53 in discrete cytoplasmic bodies (71). However, this is not

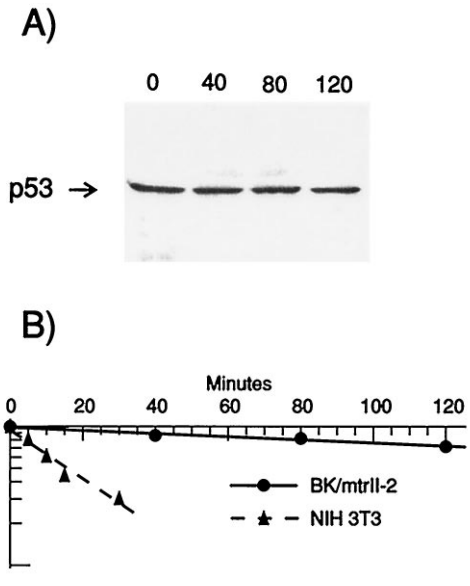

FIG. 8. Increased half-life of p53 in mtrII-expressing cells. Cell extracts of NIH 3T3 and BK/mtrII-2 cells, treated with 5 μg of cycloheximide, were prepared at the indicated times (in minutes). (A) Western blot analysis of BK/mtrII-2 cell extracts probed with anti-p53 antibody Ab-1. (B) Stability of p53 in BK/mtrII-2 cells and NIH 3T3 cells. Relative p53 levels were calculated from densitometer readings of the autoradiograph. The half-life was calculated by linear regression analysis of the curves.

the only mechanism by which adenovirus E1B proteins inhibit p53. Although adenovirus type 12 E1B binds to p53 and inhibits its p53 transactivation, it does not sequester p53 in the cytoplasm (69). Binding of adenovirus type 12 E1B to p53 does not interfere with the ability of p53 to bind specifically to DNA, and an adenovirus type 12 E1B-Gal4 fusion protein can function as a potent transcriptional repressor when brought to heterologous promoters bearing Gal4 binding sites (70). It was proposed that E1B, uniquely, can be brought to a promoter through its interaction with p53 and then act as a direct transcriptional repressor. In the case of mtrII, its binding to p53 could interfere with the transcriptional activation domain. However, this inhibition could result from an indirect effect of mtrII.

Full-length and TTL_{49} truncated mtrII proteins bound to p53 while TTL_{24} did not (Fig. 5). This correlated with results of p53-activated transcription and transformation studies. In transient-cotransfection experiments, mutant mtrII-TTL_{49} was almost as effective as the wild type in inhibiting p53-activated transcription only at a high concentration, whereas TTL_{24} had only a minimal effect. The minimal inhibition of p53-activated transcription observed with 5.0 μg of pCHC/mtrII-TTL_{24} may be due to a minimal amount of binding to p53 which may occur in the more native condition within a cell than in an in vitro immunoprecipitation experiment. The minimal inhibition observed was not due to a DNA mass effect, because no inhibition was observed after cotransfection of 2.0 μg of p53G5BCAT and 0.2 μg of pCMV/p53 with 5.0 μg of pCMV/Rb (data not shown). Importantly, both wild-type and TTL_{49} mutant mtrII-transformed rodent cells were tumorigenic in nude mice, whereas mutant TTL_{24}-transformed cells were not (59). Taken together, these results suggest an association between mtrII binding to p53, mtrII inhibition of p53 function, and mtrII tumorigenic potential.

Elevated p53 levels were detected in HCMV-infected HEL cells (38) and in HCMV-infected smooth muscle cells (57). Furthermore, p53 coimmunoprecipitated with IE2 in baculovirus-infected sf-9 cells expressing p53 and HCMV IE2. Speir et al. (57) suggested that IE2 was responsible for the up-regulation of p53. However, the data presented here suggest that mtrII could be responsible for the increased levels of p53 observed after HCMV infection.

The binding of p53 by the mtrII oncoprotein, as demonstrated in this study, may alter other functions of p53 in the cell. The wild-type p53 gene can exhibit both growth and transformation suppressor activities which result in a G_1 block in the cell cycle (26). The transcriptional transactivator function of p53 is required for growth arrest (8). Furthermore, p53 has been shown to block growth arrest in SAOS-2 cells, which lack the Rb gene (8). While p53 transactivated WAF1/Cip1 (12), GADD45 (25), mdm-2 (3, 6), and the human *bax* gene (37), it also repressed basal transcriptional machinery and certain viral promoters (17, 54, 58). p53 is also involved in DNA replication and repair processes (9, 55) and mediated DNA damage-induced apoptosis (31). Thus, disruption of p53 function by either mutation or binding to other proteins has been implicated in several human cancers (19, 33).

The current studies demonstrate the specific binding of the HCMV mtrII oncoprotein to p53 and the stabilization of p53 protein in mtrII-transformed cells with concomitant inhibition of p53-activated transcription. The investigations provide new data to suggest that human herpesvirus oncoprotein mtrII transforms cells by interacting with the cellular tumor suppressor protein p53.

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