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An N-terminal Segment of the Active Component of the Bacterial Genotoxin Cytolethal Distending Toxin B (CDTB) Directs CDTB into the Nucleus*

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Shuichi Nishikubo^{‡§}, Masaru Ohara[‡], Yoko Ueno[‡], Masae Ikura[‡], Hidemi Kurihara[§],
Hitoshi Komatsuzawa[‡], Eric Oswald[¶], and Motoyuki Sugai^{‡||}

From the Departments of [‡]Bacteriology and [§]Periodontal Medicine, Hiroshima University Graduate School of Biomedical Sciences, 1-2-3 Kasumi Minami-ku, Hiroshima 734-8553, Japan and [¶]UMR 1225 Institut National de la Recherche Agronomique "Interactions Hotes-Agents Pathogenes," Ecole Nationale Veterinaire de Toulouse, 23 chemin des Capelles, 31076 Toulouse cedex, France

Cytolethal distending toxin (CDT), produced by *Actinobacillus actinomycetemcomitans*, is a putative virulence factor in the pathogenesis of periodontal diseases. It is a cell cycle specific inhibitor at the G₂/M transition. CDTB, one of the subunits of the CDT holotoxin, is implicated in a genotoxic role after entering the target cells, whereby chromosomal damage induces checkpoint phosphorylation cascades. CDTB microinjected into the cytoplasm was shown to localize in the nucleus and induce chromatin collapse. To investigate the molecular mechanism involved in nuclear transport of CDTB, we used transient expression and microinjection of a CDTB-green fluorescent protein (GFP) fusion protein. After microinjection, His-tagged CDTB-GFP entered the nucleus in 3–4 h. Leptomycin B did not increase the speed of entry of the fusion protein, suggesting that the relatively slow entry of the fusion protein is not due to the CRM1-dependent nuclear export of the protein. Nuclear localization of the CDTB-GFP was temperature-dependent. An *in vitro* transport assay demonstrated that the nuclear localization of CDTB is mediated by active transport. An assay using transient expression of a series of truncated CDTB-GFP fusion proteins revealed that residues 48–124 constitute the minimum region involved in nuclear transport of CDTB. A domain swapping experiment of the region involved in nuclear transport of CDTB with an SV40 T nuclear localization signal indicated that CDTB is composed of two domains, an N-terminal domain for nuclear transport and a C-terminal active domain. Our results strongly suggest that nuclear localization of CDTB is required for the holotoxin to induce cytodistension and cell cycle block. This is the first demonstration that a bacterial toxin possessing a unique domain for nuclear transport is transferred to the animal cell nucleus by active transport.

Cytolethal distending toxin (CDT)¹ is a unique bacterial toxin that induces cell cycle arrest of cultured cells in the G₂ phase. It has been identified in several pathogenic bacteria including *Campylobacter* spp., *Escherichia coli*, *Shigella dysenteriae*, *Haemophilus ducreyi*, *Helicobacter hepaticus*, and *Actinobacillus actinomycetemcomitans*. The cells intoxicated with CDT show a cytopathic effect and distension in cell size, and eventually they die, which has been shown to be a common consequence of CDT treated cells (1–4). CDT holotoxin is composed of CDTA, -B, and -C, encoded by the *cdtA*, *cdtB*, and *cdtC* genes tandemly located on the *cdt* locus (1, 5–7). The CDT-induced G₂ arrest has been ascribed to the inactivation of the Cdc2-cyclin B complex, which is a key molecule for the progression of the cell cycle. In normal cells, dephosphorylation of the Thr-14 and Tyr-15 in Cdc2 triggers G₂/M transition in the cell cycle. CDT-treated cells were found to maintain Cdc2 with these residues phosphorylated in the Cdc2-cyclin B complex (8). This is because of the recruitment of Cdc25C, a Cdc2-specific phosphatase, from the nucleus to cytoplasm, which prevents dephosphorylation of the Cdc2-cyclin B complex in the nucleus (8–12). Cdc25C is regulated by Checkpoint kinase 1 or 2, which are controlled by ATM or ATR (ataxia-telangiectasia mutated and Rad3-related) (13). Recently, two research groups indicated independently that CDT has structural homology to human DNase I and suggested that CDTB is an active component of the CDT complex acting as a DNase. In support of this, *E. coli* CDTB has been demonstrated to possess nicking activity toward purified plasmid *in vitro* (14). These findings have raised the possibility that CDTB directly damages chromosomal DNA, which results in the onset of phosphorylation of the checkpoint control cascade described above. Lara-Tajero and Galan (15) demonstrated that transiently expressed or microinjected *Campylobacter jejuni* CDTB in the cultured cell cytoplasm accumulates in the nucleus, consistent with a possible nuclear function of CDTB. However, no biochemical information is available for the mechanism of nuclear accumulation of CDTB component.

In eukaryotic cells, a number of cellular proteins that function in the nucleus are imported or exported through the nu-

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|| To whom correspondence should be addressed. Tel.: 81-82-257-5635; Fax: 81-82-257-5639; E-mail: sugai@hiroshima-u.ac.jp.

¹ The abbreviations used are: CDT, cytolethal distending toxin; PI, propidium iodide; GST, glutathione S-transferase; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; NLS, nuclear localization signal; NPC, nuclear pore complex; aa, amino acid(s); Ni-NTA, nickel-nitrilotriacetic acid; ATM, ataxia telangiectasia mutation; PBS, phosphate-buffered saline; T-DNA, tumor-inducing DNA; FITC, fluorescein isothiocyanate.

TABLE I
Strains, plasmids, and primers used in this study

Strain	Plasmid	Character	Used primer	Reference
<i>Actinobacillus</i>				
standard strain				
<i>actinomycetemcomitans</i> Y4				
<i>E. coli</i> expression vector				
XL-I blue	pTK3022	<i>cdtABC</i> in pUC19 (<i>Sma</i> I- <i>Eco</i> RI site)		2
HMS174 (DE3)	pET- <i>cdtB</i> (WT)	<i>cdtB</i> (WT) in pET28a (<i>Bam</i> HI- <i>Eco</i> RI site)	<i>mdtB</i> fBg , L-2187	This study
XL-II	pFlag- <i>cdtB</i> (WT)	<i>cdtB</i> (WT) in pFlag-Mac (<i>Eco</i> RI- <i>Bgl</i> II site site)		This study
HMS174 (DE3)	pET- <i>cdtB</i> - <i>gfp</i>	<i>cdtB</i> - <i>gfp</i> in pET28a (<i>Eco</i> RI- <i>Bam</i> HI site)		This study
HMS174 (DE3)	pET- <i>gfp</i>	<i>gfp</i> in pET28a (<i>Bam</i> HI- <i>Eco</i> RI site)		This study
XL-II	pGEX-SV40 T nls - <i>gfp</i>	SV40 T nls - <i>gfp</i> in pGEX-2T (<i>Bam</i> HI- <i>Eco</i> RI site)		Y. Yoneda
HMS174 (DE3)	pET- <i>cdtB</i> Δ 48-124 - SV40 T nls	<i>cdtB</i> Δ 48-124 - SV40 T nls in pET28a (<i>Bam</i> HI- <i>Eco</i> RI site)	<i>mdtB</i> fBg , L-2187, U-1476 <i>cdtB</i> -SV40 chimera, L-1476 <i>cdtB</i> -SV40 chimera, U-1723 <i>cdtB</i> -SV40 chimera, L-1723 <i>cdtB</i> -SV40 chimera	This study
M15	pQE- <i>cdtABC</i> (WT)	<i>cdtABC</i> (WT) in pQE60 (<i>Nco</i> I- <i>Bgl</i> II site)	QIA-CDTA-U2, QIA-CDTC-L	This study
M15	pQE- <i>cdtAB</i> Δ 11aa C	<i>cdtAB</i> Δ 11aa C in pQE60 (<i>Nco</i> I- <i>Bgl</i> II site)	QIA-CDTA-U2, QIA-CDTC-L, U-nls C term 11aa, L-nls C	This study
M15	pQE- <i>cdtAB</i> Δ 11aa+SV40 T nls C	<i>cdtAB</i> Δ 11aa+SV40 T nls C on pQE60 (<i>Nco</i> I- <i>Bgl</i> II site)	term 11aa QIA-CDTA-U2, QIA-CDTC-L, U-f 11aa+SV40NLS, L-f	This study
M15	pQE- <i>cdtAB</i> Δ 11aa C	<i>cdtAB</i> Δ 11aa C in pQE60 (<i>Nco</i> I- <i>Bgl</i> II site)	11aa+SV40NLS, U-r 11aa+SV40NLS, L-r 11aa+SV40NLS QIA-CDTA-U2, QIA-CDTC-L, U-nls C term 11aa, L-nls C	This study
M15	pQE- <i>cdtAB</i> Δ 11aa+SV40 T nls C	<i>cdtAB</i> Δ 11aa+SV40 T nls C on pQE60 (<i>Nco</i> I- <i>Bgl</i> II site)	term 11aa QIA-CDTA-U2, QIA-CDTC-L, U-f 11aa+SV40NLS, L-f 11aa+SV40NLS, U-r 11aa+SV40NLS, L-r 11aa+SV40NLS	This study
Mammalian expression vector				
XL-II	pEGFP- <i>cdtB</i>	<i>cdtB</i> in pEGFP-C1 (<i>Bgl</i> II- <i>Eco</i> RI site)	<i>mdtB</i> fBg , L-2187	This study
XL-II	pEGFP- <i>cdtB</i> 23-200	534bp deletion fragment of <i>cdtB</i> in pEGFP-C1 (<i>Bgl</i> II- <i>Eco</i> RI site)	<i>mdtB</i> fBg , <i>Aa</i> spe3'	This study
XL-II	pEGFP- <i>cdtB</i> 167-283	753bp deletion fragment of <i>cdtB</i> in pEGFP-C1 (<i>Bgl</i> II- <i>Eco</i> RI site)	U-1851, L-2187	This study
XL-II	pEGFP- <i>cdtB</i> 23-154	396bp deletion fragment of <i>cdtB</i> in pEGFP-C1 (<i>Bgl</i> II- <i>Eco</i> RI site)	<i>mdtB</i> fBg , L-1812	This study
XL-II	pEGFP- <i>cdtB</i> 23-93	213bp deletion fragment of <i>cdtB</i> in pEGFP-C1 (<i>Bgl</i> II- <i>Eco</i> RI site)	<i>mdtB</i> fBg , L-1629	This study
XL-II	pEGFP- <i>cdtB</i> 23-59	111bp deletion fragment of <i>cdtB</i> in pEGFP-C1 (<i>Bgl</i> II- <i>Eco</i> RI site)	<i>mdtB</i> fBg , L-1527	This study
XL-II	pEGFP- <i>cdtB</i> 83-154	186bp deletion fragment of <i>cdtB</i> in pEGFP-C1 (<i>Bgl</i> II- <i>Eco</i> RI site)	U-1629fBg , L-1812	This study
XL-II	pEGFP- <i>cdtB</i> 83-154	216bp deletion fragment of <i>cdtB</i> in pEGFP-C1 (<i>Bgl</i> II- <i>Eco</i> RI site)	U-1596fBg , L-1812	This study
XL-II	pEGFP- <i>cdtB</i> 79-154	228bp deletion fragment of <i>cdtB</i> in pEGFP-C1 (<i>Bgl</i> II- <i>Eco</i> RI site)	U-1584fBg , L-1812	This study
XL-II	pEGFP- <i>cdtB</i> 72-154	249bp deletion fragment of <i>cdtB</i> in pEGFP-C1 (<i>Bgl</i> II- <i>Eco</i> RI site)	U-1564fBg , L-1812	This study
XL-II	pEGFP- <i>cdtB</i> 48-154	321bp deletion fragment of <i>cdtB</i> in pEGFP-C1 (<i>Bgl</i> II- <i>Eco</i> RI site)	U-1491fBg , L-1812	This study
XL-II	pEGFP- <i>cdtB</i> 41-154	342bp deletion fragment of <i>cdtB</i> in pEGFP-C1 (<i>Bgl</i> II- <i>Eco</i> RI site)	U-1467fBg , L-1812	This study
XL-II	pEGFP- <i>cdtB</i> 23-142	360bp deletion fragment of <i>cdtB</i> in pEGFP-C1 (<i>Bgl</i> II- <i>Eco</i> RI site)	<i>mdtB</i> fBg , L-1776	This study
XL-II	pEGFP- <i>cdtB</i> 23-134	336bp deletion fragment of <i>cdtB</i> in pEGFP-C1 (<i>Bgl</i> II- <i>Eco</i> RI site)	<i>mdtB</i> fBg , L-1752	This study
XL-II	pEGFP- <i>cdtB</i> 23-124	306bp deletion fragment of <i>cdtB</i> in pEGFP-C1 (<i>Bgl</i> II- <i>Eco</i> RI site)	<i>mdtB</i> fBg , L-1722	This study
XL-II	pEGFP- <i>cdtB</i> 23-102	240bp deletion fragment of <i>cdtB</i> in pEGFP-C1 (<i>Bgl</i> II- <i>Eco</i> RI site)	<i>mdtB</i> fBg , L-1656	This study
XL-II	pEGFP- <i>cdtB</i> 48-124	231bp deletion fragment of <i>cdtB</i> in pEGFP-C1 (<i>Bgl</i> II- <i>Eco</i> RI site)	U-1491fBg , L-1722	This study
XL-II	pEGFP- <i>cdtB</i> 125-283	477bp deletion fragment of <i>cdtB</i> in pEGFP-C1 (<i>Bgl</i> II- <i>Eco</i> RI site)	U-1723fBg , L-2187	This study
XL-II	pEGFP- <i>cdtB</i> Δ 11aa	<i>cdtB</i> Δ 11aa in pEGFP-C1 (<i>Bgl</i> II- <i>Eco</i> RI site)	<i>mdtB</i> fBg , L-2187	This study
XL-II	pEGFP- <i>cdtB</i> Δ 11aa+SV40 T nls	<i>cdtB</i> Δ 11aa+SV40 T nls in pEGFP-C1 (<i>Bgl</i> II- <i>Eco</i> RI site)	<i>mdtB</i> fBg , L-2187	This study
Primer	Position*	Sequence	Restriction enzyme site	Reference
<i>mdtB</i> fBg	1414-1425	5'-CAAGATCTGCTAACTTGAGT-3'	<i>Bgl</i> II	This study
<i>Aa</i> spe3'	1926-1949	5'-AAATCACCAACAACCATCCAGCTA-3'		2
U-1851	1851-1875	5'-TGATGCGGTAAGTTAATTCGTAATA-3'		2
L-1323	1323-1350	5'-ATAAACTCTAGCTTAATTAACCGCTG-3'		2
L-1527	1503-1527	5'-TGCACCTTGTTCTCCGATAATAAT-3'		This study
L-1629	1605-1629	5'-CCAGGTATATTCCTCAATTGGCGTT-3'		This study
L-1812	1688-1812	5'-ATCAGTACCAATGCGGATACCTACT-3'		This study
L-1656	1633-1656	5'-ATTTGGACGGGAGCGAGTACCTAA-3'		This study
L-1722	1698-1722	5'-TGACACGATAGCTAAGTTCACCTCG-3'		This study
L-1752	1729-1752	5'-TACGATAAAAAGCTTCATCGGCTTG-3'		This study
L-1776	1753-1776	5'-TTGAAGCACAGAAGAATCAGAATG-3'		This study
L-2188	2188-2212	5'-AGTATTCCTTAGCGATCATGAA-3'		2
U-1629fBg	1629-1649	5'-AGATCTAATTAGGTACTCGCTCC-3'	<i>Bgl</i> II	This study
U-1596fBg	1596-1614	5'-AGATCTCATGGGGAACGCCAAT-3'	<i>Bgl</i> II	This study
U-1584fBg	1584-1602	5'-AGATCTCGAGTAATCAACATGGG-3'	<i>Bgl</i> II	This study
U-1564fBg	1564-1581	5'-AGATCTAGTCCGCGAGTAAGAACC-3'	<i>Bgl</i> II	This study
U-1467fBg	1467-1473	5'-AGATCTGTAATGAAAGTAAATGG-3'	<i>Bgl</i> II	This study
U-1491fBg	1491-1509	5'-AGATCTAATGTGCGCAATTATTA-3'	<i>Bgl</i> II	This study
U-1723fBg	1723-1747	5'-AGATCTCGTCTCAAGCCGATGAA-3'	<i>Bgl</i> II	This study
QIA-CDTA-U2	654-676	5'-GCCATGGTAAGGAGAGGTACAATGAAAAAG-3'	<i>Nco</i> I	This study
QIA-CDTC-L	2761-2778	5'-AAAGATCTGCTACCCCTGA-3'	<i>Bgl</i> II	This study
U-1476 <i>cdtB</i> -SV40 chimera	1476-1491	5'-AGTAAATGGAATATTCCTCCAAAAA-3'		This study
L-1476 <i>cdtB</i> -SV40 chimera	1476-1491	5'-TTTTGGAGGAATATTCATTACT-3'		This study
U-1723 <i>cdtB</i> -SV40 chimera	1723-1738	5'-AGAAGACCCCGTCTCAAGCCGAT-3'		This study

TABLE I—continued

Primer	Position*	Sequence	Restriction enzyme site	Reference
L-1723- <i>cdtB</i> -SV40 chimera	1723-1738	5'-ATCGGCTTGACGACGGGGTCTTCT-3'		This study
U- <i>nls</i> C term Δ 11aa	1677-1689, 1723-1735	5'-CGTTTATAGATGTCGTCGTAAGCC-3'		This study
L- <i>nls</i> C term Δ 11aa	1677-1689, 1723-1735	5'-GGCTTGACGACGAACATCTAAACG-3'		This study
U- Δ 11aa+SV40NLS	1677-1689	5'-CGTTTATAGATGTCCTCCAAAAAAG-3'		This study
L- Δ 11aa+SV40NLS	1677-1689	5'-CTTTTTGGAGGAACATCTAAACG-3'		This study
U- Δ 11aa+SV40NLS	1723-1735	5'-GTAGAAGACCCCGTCGTAAGCC-3'		This study
L- Δ 11aa+SV40NLS	1723-1735	5'-GGCTTGACGACGGGGTCTTCTAC-3'		This study

* Position corresponds to the DNA sequence published in GeneBank accession No. AB 011450.

clear pore complex (NPC), which forms a tunnel through the nuclear envelope (16–18). Although molecules smaller than 40–60 kDa pass by diffusion through the NPC, most of the macromolecules are generally carried by energy-dependent active transport. Such nuclear imported proteins have common features. For example they possess a conserved nuclear localization signal (NLS), which allows their rapid import via complex formation with carrier protein families in the cells. The classical monopartite or bipartite NLSs are characterized as lysine- or arginine-rich sequences, which bind to the carrier protein, importin- α . There are some variations of NLS that do not possess conserved basic amino acid residues, such as NLS in M9 (19) or the RPA protein (replication protein A) (20). They are called atypical NLS, and the number of reports on atypical NLS are increasing (21, 22).

Herein, we demonstrate that the *A. actinomycetemcomitans* CDTB component is transferred to the nucleus by active transport. Furthermore, we have defined a unique and functional domain in the N-terminal segment of CDTB component that mediates nuclear import. We also demonstrated that nuclear entry of CDTB is required for the holotoxin to show cytodistension and cell cycle block. These studies strongly indicate that CDTB is a nuclear targeting genotoxin using the eukaryotic active transport system.

EXPERIMENTAL PROCEDURES

Cell Culture and Plasmids—HeLa cells (ATCC CCL2) and other mammalian cells were cultured in Dulbecco's modified Eagle's medium (Nissui) supplemented with 10% calf serum at 37 °C in a 5% CO₂, 95% air atmosphere. Plasmids and bacteria used in this study are listed in Table I. All *E. coli* were laboratory strains and grown aerobically in Luria Bertani (LB) medium or on LB agar plates. *A. actinomycetemcomitans* was grown in Trypticase Soy Broth (BD Biosciences) supplemented with 1% (w/v) yeast extract in a 5% CO₂ atmosphere. Ampicillin (50 μ g/ml) or kanamycin (50 μ g/ml) was added when necessary.

Manipulation of DNA and Plasmid Construction—The DNA region of *A. actinomycetemcomitans* *cdtB* gene, corresponding to the signal peptide-cleaved mature form of CDTB (23–283 aa), was isolated from *A. actinomycetemcomitans* Y4 genomic DNA by PCR amplification, using specific primers containing restriction enzyme sites, for subcloning into vectors. Primers used in this study are listed in Table I. The PCR-amplified *cdtB* gene was once cloned into pGEM-T Easy PCR cloning vector (Promega) and was subcloned into the pET28a (Novagen), the histidine-tagged expression vector, or pFlag max (Kodak), the FLAG-tagged expression vector. Various *cdtB* deletion mutants were generated by PCR using primers listed in Table I. The deletion mutants were also cloned into the pGEM-T Easy and subcloned into the pEGFP-C1, green fluorescence protein fusion vector (Clontech).

Preparation of CDTB Subunit—CDTB was highly expressed in recombinant *E. coli* carrying pET-*cdtB* in the presence of 0.1 mM isopropyl-1-thio- β -D-galactopyranoside at the point of OD₆₆₀ = 0.5–0.7. After induction for 3 h at 30 °C, cells were harvested by centrifuge at 5000 \times g for 5 min. Harvested bacterial cells were washed with phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.3) twice. Cells resuspended in PBS with 1% Triton X-100 were broken by an ultrasonic disrupter (TOMY) and centrifuged at 5000 \times g for 5 min to remove unbroken cells. The supernatant was further centrifuged at 100,000 \times g to remove the membrane fraction. The resulting supernatant was incubated with nickel-chelated agarose (Ni-NTA, Qiagen). Ni-NTA metal affinity purification was carried out according to the instruction manual from Qiagen. The Ni-NTA-purified

CDT was further purified using high pressure liquid chromatography (Tosoh, Tokyo) equipped with a solvent delivery pump CCPM and UV8011 absorbance detector. A UnoS cation exchange chromatography column (Bio-Rad) was used for purification of CDTB protein. To obtain a better purification of CDTB protein, TSK gel G3000 PW (Tosoh) was used for gel permeation chromatography.

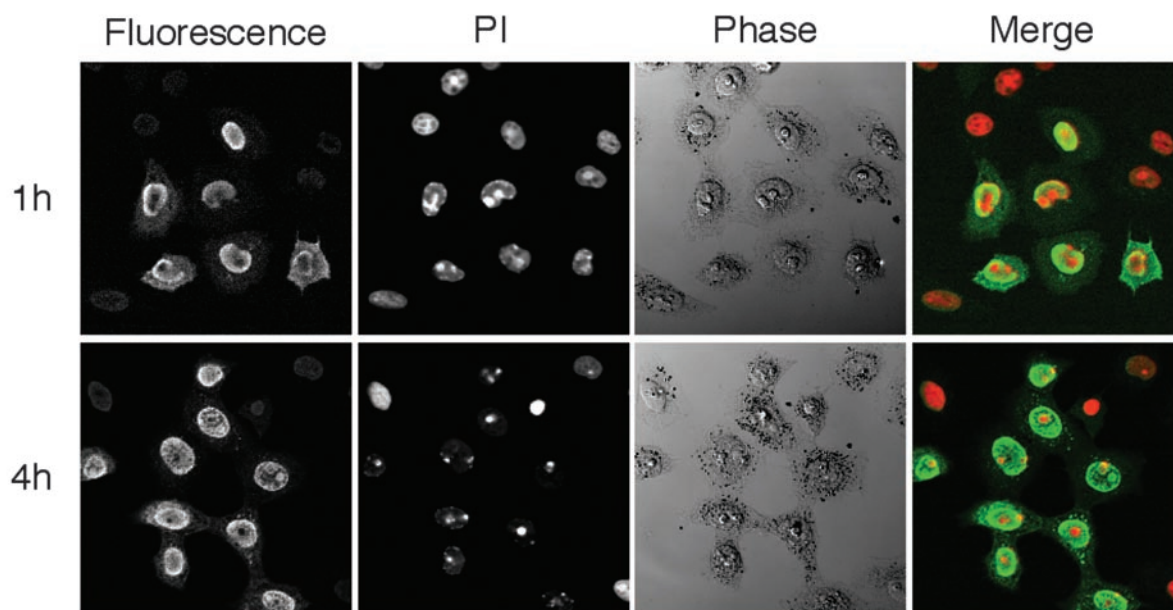
Plasmid Transfection—Plasmids were delivered into HeLa and other mammalian cells by calcium phosphate methods. Cells were prepared at 1 \times 10⁶/ml in 5 ml on 60-mm tissue culture dish (Corning). Plasmid DNA (10 μ g) was mixed with 500 μ l of H₂O and 50 μ l of 2.5 M CaCl₂ and incubated at room temperature for 10 min. Then 500 μ l of 2 \times Hepes buffer (2.8 mM Na₂HPO₄, 280 mM NaCl, 50 mM Hepes, pH 7.1) was added to the DNA solution and kept at room temperature for 10 min. All of the solution was overlaid onto the cultured cells. After incubation at 37 °C for 12 h in a 5% CO₂ incubator, cells were washed twice with PBS. 1.5 ml of glycerol-Hepes buffer (10% glycerol, 1 \times Hepes buffer) was added at room temperature for 30 s. Cells were washed twice with PBS and cultured in Dulbecco's modified Eagle medium with 10% calf serum in a 5% CO₂, 95% air atmosphere.

Microinjection—Microinjection was performed using the Eppendorf Injectman NI2. Purified proteins were adjusted to the concentration of 1 μ g/ μ l and injected into the cytosol of cultured cells at a pressure of 50–120 hpa for a duration of 0.2 s. The cells injected with CDTB fused to green fluorescence protein (GFP) were observed directly by confocal microscopy (Carl Zeiss LSM 401). Cells without fluorophore were stained by immunohistochemistry. Briefly cells were washed three times with PBS and fixed with 3.7% formaldehyde, 0.5% Triton X-100 in PBS for 10 min at room temperature. Cells were again washed three times with PBS and added with 1% bovine serum albumin in PBS (blocking buffer) at room temperature for 10 min. The primary antibody in blocking buffer was overlaid on cells and kept at room temperature for 40 min. Cells were washed three times with PBS, and the secondary antibody conjugated with fluorescein isothiocyanate or rhodamine in blocking buffer was added and incubated at room temperature for 30 min. After being washed with PBS three times, the cells were incubated with propidium iodide (PI) in PBS (5 μ g/ml) for 5 min at 37 °C. After being washed again with PBS three times, the cells were observed by confocal microscopy.

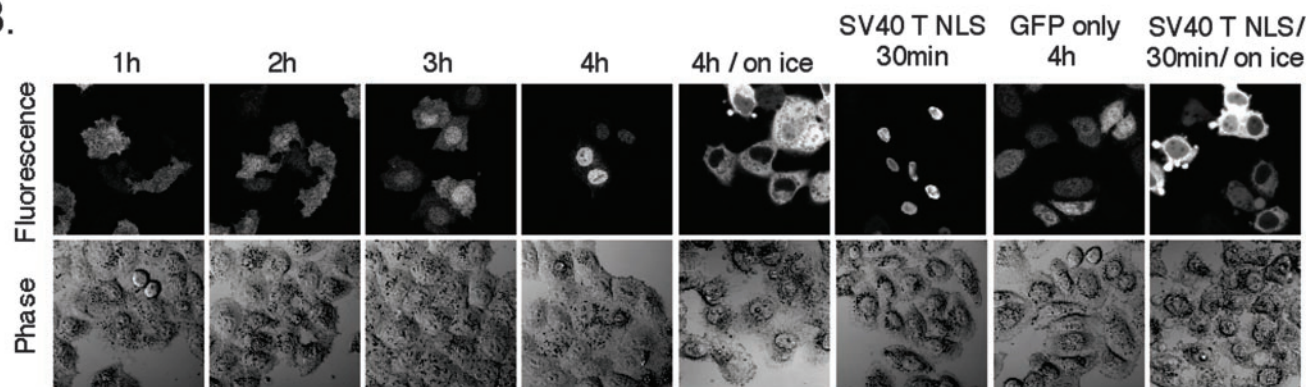
Cytosol Preparation—Cytosol was prepared by the method described by Miyamoto *et al.* (23). HeLa cells were harvested by scraping in PBS and pelleted by centrifuge at 5000 \times g for 5 min. Cells were washed in 5 ml of washing buffer (10 mM Hepes, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 2 mM dithiothreitol), by centrifuge at 5000 \times g for 5 min. Cells were then resuspended in lysis buffer (5 mM Hepes, pH 7.3, 10 mM potassium acetate, 2 mM magnesium acetate, 2 mM dithiothreitol, 20 μ M cytochalasin B, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin), kept on ice for 10 min, and homogenized for 5–10 strokes with a Dounce homogenizer. Lysed cells were centrifuged at 1,500 \times g for 15 min. The supernatant was further centrifuged at 100,000 \times g for 30 min, and the resultant supernatant was used as cytosol.

In Vitro Transport Assay—An *in vitro* transport assay was carried out by the method described by Nagoshi and Yoneda (24). HeLa cells (5 \times 10⁶/ml) grown overnight were washed twice with transport buffer (20 mM Hepes, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 5 mM sodium acetate, and 0.5 mM EGTA), and digitonin was added at a concentration of 40 μ g/ml. Cells were permeabilized by being kept on ice for 5 min and washed with transport buffer containing 2 mM dithiothreitol, 1 μ g/ml leupeptin, and 1 μ g/ml pepstatin. Permeabilized cells were overlaid with 1 μ g/ μ l extracted cytosol, 0.1 mM ATP, 0.5 mM phosphocreatine, 2 units/ml creatine kinase, 0.05 mM GTP, and the protein of interest. The *in vitro* transport assay was carried out by incubating cells at 30 °C for an appropriate time. Cells were washed twice with TB containing 2 mM dithiothreitol, 1 μ g/ml leupeptin, and 1 μ g/ml pepstatin and then fixed with 3.7% formaldehyde in TB for 10 min.

A.



B.



C.

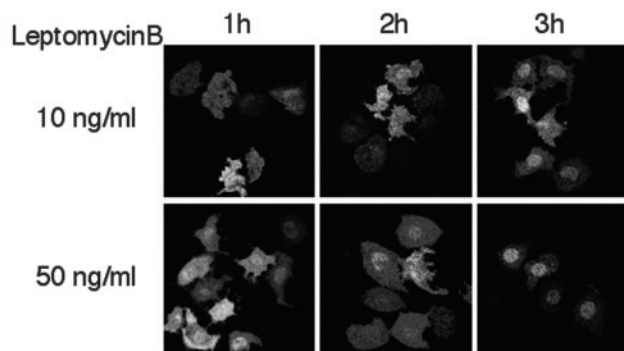


FIG. 1. Microinjected CDTB enters into HeLa nucleus. *A*, purified His-CDTB was microinjected into HeLa cells, and His-CDTB was detected by immunostaining using rabbit anti-CDTB serum as primary antiserum followed by fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG goat serum as secondary antiserum. Wavelengths of 488 nm and 543 nm were used to excite FITC and PI, respectively. Emission spectra were collected with 510–525-nm band-pass filters and 570-nm long-pass filters. Computer-generated overlays of the fields in the fluorescence (*green*) and those of PI (*red*) are shown (*Merge*). The fluorescent signal began to be detected in the nucleus 30 min after microinjection. The chromatin stained with PI shows the decreased signal at 4 h. *B*, His-CDTB-GFP, which has a molecular mass of ~65.6 kDa, was purified by a nickel-affinity column and microinjected into HeLa cells. Localization of microinjected protein was monitored every hour after microinjection by a laser confocal microscopy. GST-SV40 T NLS-GFP and GFP were used as positive and negative controls, respectively. *C*, leptomycin B, a specific inhibitor of CRM1/exportin 1, was added to the HeLa cells at 0, 10, and 50 ng/ml, 4 h before CDTB-GFP microinjection. Localization of microinjected protein was monitored every hour after microinjection by confocal microscopy.

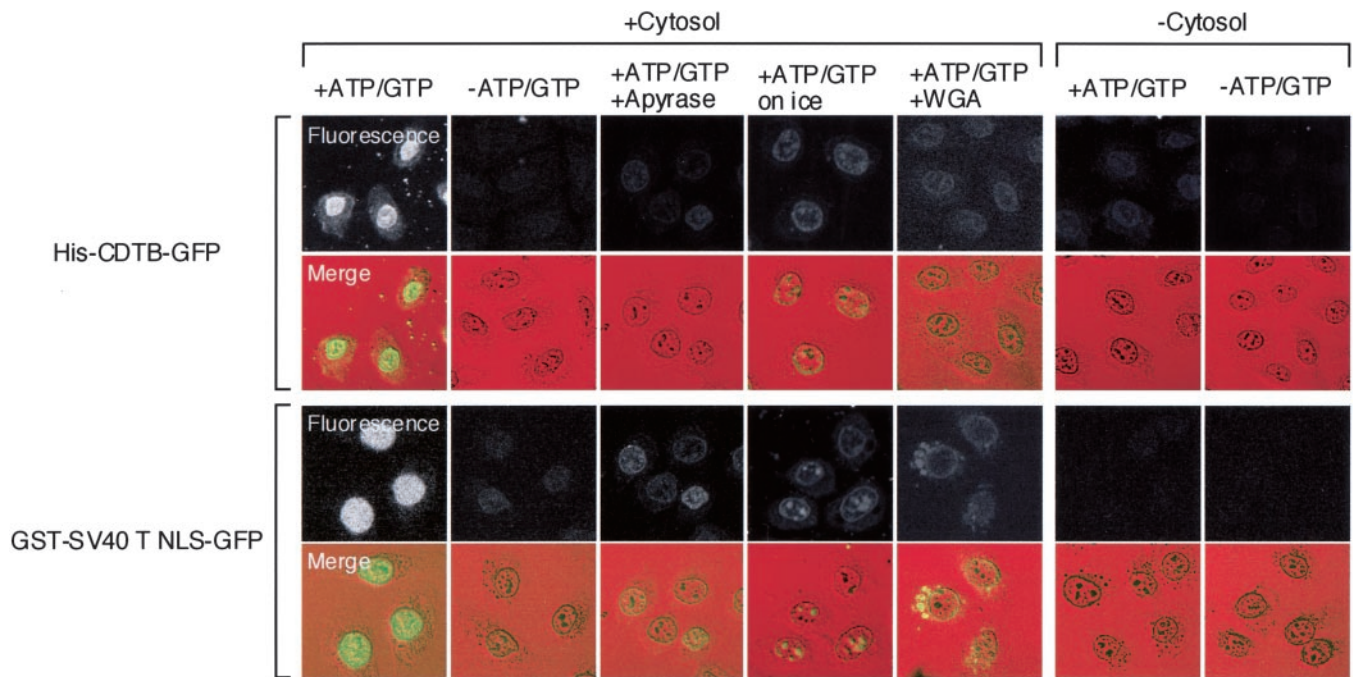


FIG. 2. His-CDTB-GFP passes through NPC and requires energy for its nuclear localization. The *in vitro* transport assay was carried out using digitonin-permeabilized HeLa cells with cytosol extract and energy-regenerative solution. Import of CDTB-GFP was analyzed by confocal microscopy using the FITC channel signal (Fluorescence). Computer-generated overlays of the fields in the fluorescence (green) and those of phase contrast microscopy (red) are shown (Merge). His-CDTB-GFP was transported into the nucleus in the presence of cytosol and ATP/GTP solution. The addition of apyrase, an ATP hydrolase, or incubation at 4 °C prevented the entry of His-CDTB-GFP into the nucleus. Wheat germ agglutinin (WGA), a specific inhibitor of NPC, inhibited nuclear transport of His-CDTB-GFP.

Other Procedures—The cell cycle was analyzed by FACSscan (FACS-Calibur, BD Biosciences) after staining cells with PI as described (2). Crude CDT holotoxin was prepared from the periplasmic space in *E. coli* possessing pTK3022 (2). Routine DNA and protein manipulations were performed by standard procedures. All restriction enzymes and T4 DNA ligase were from Roche Applied Science or New England Biolabs (Beverly, MA). Other materials and chemicals used were from commercial sources. SV40 T NLS was expressed in recombinant *E. coli* carrying pGEX-SV40nls-gfp and purified using a glutathione-Sepharose column.

RESULTS

CDTB Microinjected into Cytosol Translocates to the Nucleus—To know the final destination of the functional CDTB subunit, we first attempted to microinject the His-CDTB protein into the cytoplasm of cultured cells and trace its moiety. Microinjection of His-CDTB and subsequent immunostaining of the cells with anti-CDTB serum showed the nuclear localization of His-CDTB and confirmed the observation made on *Campylobacter* CDT (15) (Fig. 1A). Time course measurement of His-CDTB localization after microinjection revealed that His-CDTB entered into the nucleus 30 min after microinjection and remained *in situ* as late as 4 h (Fig. 1B). We simultaneously stained the cells with PI to observe the chromatin. PI staining showed chromatin disintegration at 4 h, but not at 1 h, suggesting that microinjected CDTB acts as a DNase or indirectly induces DNA injury. To exclude the possibility that His₆ worked as a nuclear localization signal, we constructed the plasmid pFlag-cdtB to produce the FLAG-tagged CDTB and purified it by anti-FLAG antibody affinity chromatography. Microinjected FLAG-tagged CDTB also localized in the nucleus of HeLa cells by the detection of immunostaining using anti-CDTB serum (not shown). These results clearly indicated that *A. actinomycetemcomitans* CDTB, by itself, has an ability to localize in the nucleus.

Nuclear Localization of CDTB Is Mediated by Active Transport—To exclude a possibility that CDTB is transported through the NPC by diffusion because of its relatively small molecular mass (28 kDa), we tried to use a fusion protein. We

constructed a plasmid, pET-cdtB-gfp, that produces His-tagged CDTB-green fluorescence protein fusion (His-CDTB-GFP), which has a predicted molecular mass of 65.6 kDa. His-CDTB-GFP can be monitored directly by confocal microscope without fixation or antibody reaction. After purification by affinity chromatography, His-CDTB-GFP was microinjected into the cytoplasm of HeLa cells. GST-SV40 T NLS-GFP, which is imported into the nucleus by a classical monopartite NLS (PP-KKKRKVEDP), was used as a positive control. As shown in Fig. 1B, microinjected GST-SV40 T NLS-GFP was imported into the nucleus in 30 min. Microinjected His-CDTB-GFP was also transported into the nucleus with relatively slow rate. After microinjection, His-CDTB-GFP was detected in the nucleus from as early as 2 h and apparently as late as 3 h, whereas GFP alone did not accumulate in the nucleus even after 4 h. It should be noted that even after 4 h the microinjected cells exhibited a fairly weak cytoplasmic staining besides the strong nuclear staining. These phenomena of His-CDTB-GFP localization could be due to nuclear export of the protein. To address this possibility, cells were treated with an inhibitor of the CRM1-exportin pathway, leptomycin B (25), and the subcellular localization of microinjected His-CDTB-GFP was assessed. Leptomycin B was added to the cells prior to microinjection and localization of His-CDTB-GFP was monitored up to 4 h. As shown in Fig. 1C, there was no difference in the import rate and the staining of the nucleus and cytoplasm irrespective of the presence or absence of leptomycin B. These data suggest that these phenomena do not result from CRM1-dependent nuclear export of His-CDTB-GFP. We next assessed the temperature dependence of His-CDTB-GFP nuclear transport. Incubation of cells on ice suddenly after microinjection prevented the transport of GST-SV40 T NLS-GFP into the nucleus as expected (Fig. 1B). Similarly, the nuclear transport of His-CDTB-GFP was prevented when the microinjected cells were placed on ice (Fig. 1B). The temperature dependence of His-CDTB-GFP nuclear localization prompted us to investigate

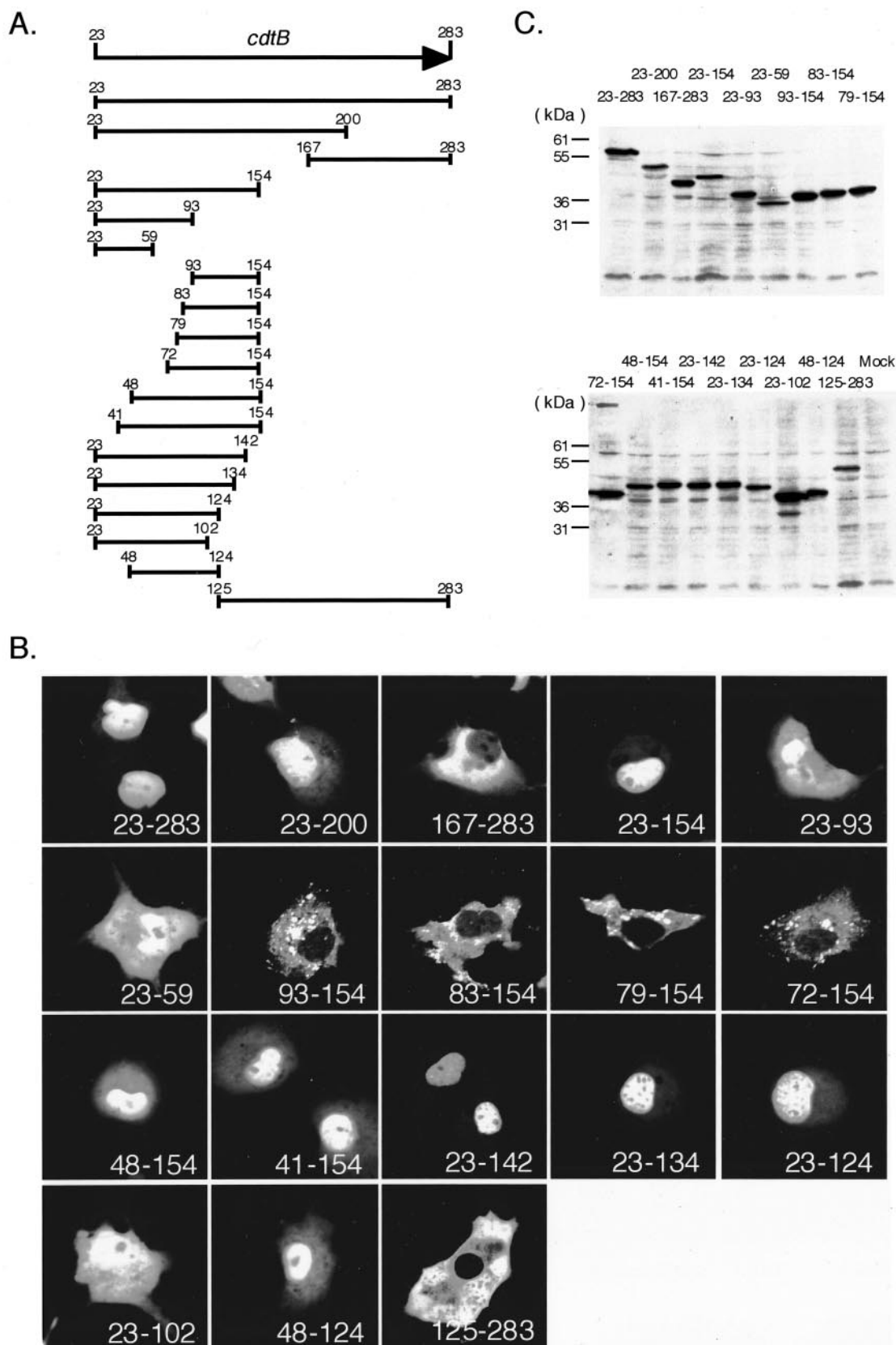


FIG. 3. Identification of region involved in nuclear transport of CDTB. A series of *cdtB* deletions was cloned into pEGFP in-frame to express various EGFP-deleted CDTB mutants. Each recombinant plasmid was transfected into HeLa cells, and localization of green fluorescence was monitored by a laser confocal microscope. **A**, schematic representation of deletion constructs. **B**, green fluorescence of deletion clone transiently expressed in COS7 cells. **C**, immunoblotting analysis of homogenate of COS7 cells transiently expressed EGFP-deleted CDTB mutants. Anti-EGFP rabbit antibody was used as the primary antibody. Immunodetection was performed as described elsewhere (2). **D**, alignment of CDTB sequences from a variety of bacteria. Amino acid sequences of CDTB from various bacteria and mouse DNase I are aligned using the Clustal W program. The identical amino acids are boxed in gray. The domain for nuclear transport is indicated by a bold underline. The conserved catalytic amino acids are boxed with a red line. The conserved metal binding sites are boxed with a blue line with an asterisk underneath. *Actinobacillus actinomyce-*

D.

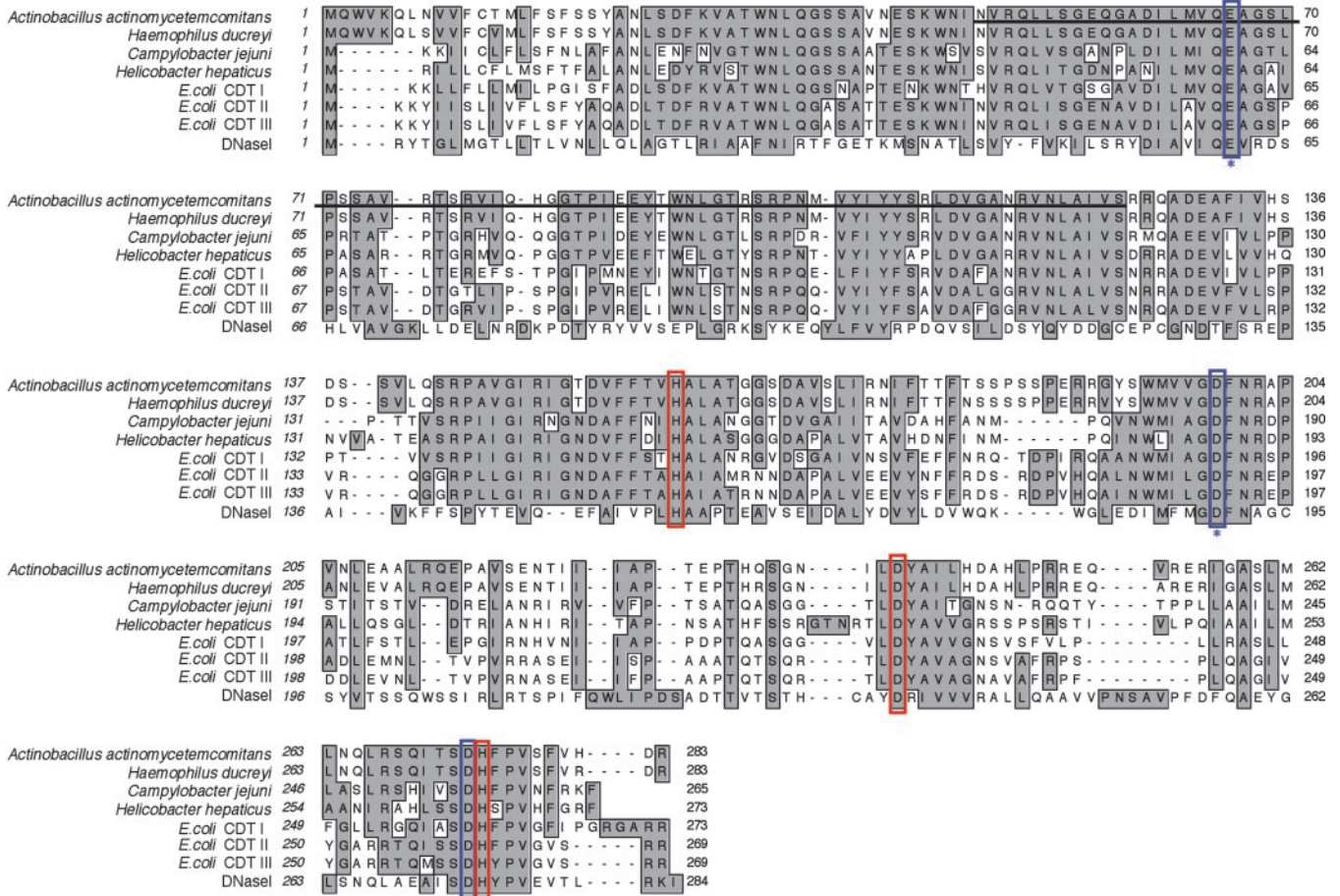


Fig. 3—continued

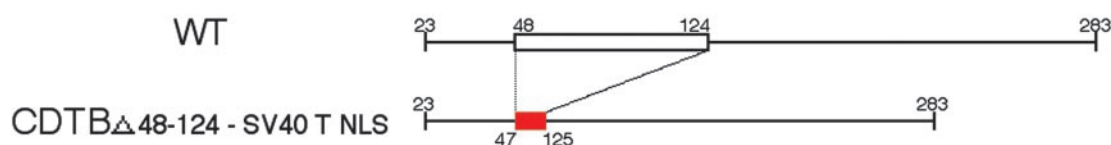
the energy requirement for the nuclear import. We used the *in vitro* transport assay to identify the molecules necessary for CDTB-nuclear import. Digitonin-permeabilized HeLa cells were overlaid with a solution containing His-CDTB-GFP, HeLa cell cytosol, energy regeneration components (ATP, GTP, phosphocreatine, and creatine kinase), and incubated at 30 °C for 4 h. As a control, GST-SV40 T NLS-GFP was used. The nuclear import of GST-SV40 T NLS-GFP has been shown previously to be energy-dependent, and we were able confirm these results (Fig. 2). Similarly, His-CDTB-GFP was able to enter into the nucleus in the presence of the cytosol extract and energy resources. The temperature dependence of the nuclear transport of His-CDTB-GFP was again confirmed using the *in vitro* transport system. In the absence of GTP/ATP, His-CDTB-GFP failed to enter into the nucleus. Treatment with apyrase, one of the ATP hydrolases, also abolished the entry of both His-CDTB-GFP and GST-SV40 T NLS-GFP into the nucleus. Wheat germ agglutinin is a lectin that presumably inhibits nuclear protein transport by interacting with the nuclear pore complex protein with *O*-linked *N*-acetylglucosamine moieties (26, 27). When the permeabilized HeLa cells were preincubated

with wheat germ agglutinin, nuclear entry of both His-CDTB-GFP and GST-SV40 T NLS-GFP was inhibited. These results strongly suggested that nuclear import of CDTB required energy and that the His-CDTB-GFP passes through the nuclear pore complex just like other mammalian nuclear proteins.

N-terminal Stretch of CDTB (48–124 aa) Is Responsible for Nuclear Localization—Because the amino acid sequence of CDTB does not contain a typical nuclear localization signal, it was of interest whether CDTB possesses functional region(s) involved in nuclear transport in the molecule. A preliminary trial to purify a series of truncated CDTB proteins using an *E. coli*-vector system was unsuccessful because of the instability of the recombinant products. Therefore, we switched to using direct expression of the truncated CDTB-GFP fusion protein in COS7 cells, and expression of truncated CDTB was verified by immunoblot analysis of the transfected cells (Fig. 3B). The *cdtB* gene was cloned in-frame into pEGFP-C1 and transiently transfected into COS7 cells to generate GFP fused to a mature form of CDTB (CDTB-GFP). As shown in Fig. 3C, GFP-CDTB was detected in the nucleus 4 h after plasmid transfection. In order to know the region involved in nuclear transport of

temcomitans, locus cloned from *A. actinomycetemcomitans* Y4 (GenBank™ accession no. AB011405); *Haemophilus ducreyi*, locus cloned from *H. ducreyi* 35000 (GenBank™ accession no. U53215); *Campylobacter jejuni*, locus cloned from *C. jejuni* 81–176 (GenBank™ accession no. U51121); *Helicobacter hepaticus*, locus cloned from *H. hepaticus* (GenBank™ accession no. AAF19158); *E. coli* CDT I, locus cloned from *E. coli* E6468/62 (GenBank™ accession no. U03293); *E. coli* CDT II, locus cloned from *E. coli* 9142–88 (GenBank™ accession no. U04208); *E. coli* CDT III, locus cloned from *E. coli* 1404 (GenBank™ accession no. U89305); DNase I, locus cloned from *Mus musculus* (GenBank™ accession no. AAH30394).

A.



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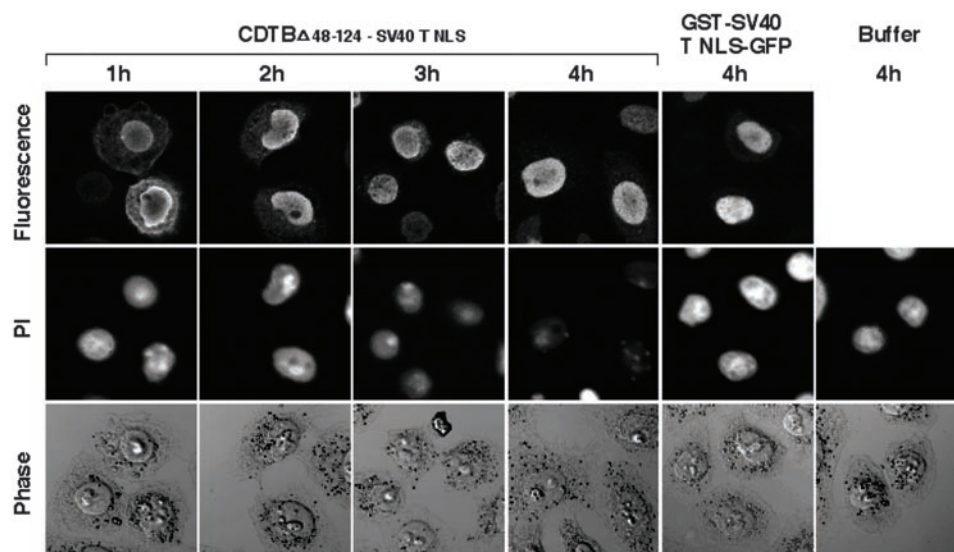


FIG. 4. Chimera protein of CDTB Δ 48–124-SV40 T NLS acts as a genotoxin in the nucleus. A, schematic representation of wild type CDTB (WT) and CDTB with its amino acid sequence (48–124 aa) exchanged with SV40 T NLS (CDTB Δ 48–124-SV40 T NLS). B, wild type CDTB and CDTB with its amino acid sequence (48–124 aa) exchanged with SV40 T NLS were purified using Ni-NTA. Purified protein was analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining. C, purified chimeric protein was microinjected into the cytosol of HeLa cells. The protein was localized by immunostaining using anti-CDTB serum. Chromatin was stained simultaneously with propidium iodide. GST-SV40 T NLS-GFP was used as a positive control.

CDTB, we first divided CDTB into two parts, the N-terminal (23–200 aa) and C-terminal (167–283 aa) domains, and constructed plasmids to express each fragment as GFP-fused protein in the cells. After plasmid transfection, localization of the GFP-fused protein was assessed. As shown in Fig. 3C, nuclear localization was observed in the cells expressing the N-terminal portion of CDTB fused to GFP but not in the cells expressing the C-terminal half fused to GFP. Therefore, we created a series of deletion constructs of the N-terminal portion of CDTB and searched for the region with which the fusion protein clearly localized into the nucleus. In some cases, the intensity of fluorescence appeared equal in both the nucleus and the cytoplasm. Finally, a region corresponding to 48–124 aa was found to be the minimum region responsible for nuclear localization of the CDTB molecule. Further deletion of either the N-terminal or C-terminal ends of this segment resulted in loss of function of the apparent nuclear localization. Similar results were obtained when HeLa cells were used to transiently express truncated CDTB-GFP fusion protein (not shown). We also tried to express GFP fused to CDTB 48–124 aa (GFP-CDTB-(48–124)) in other cell lines such as Chinese hamster ovary cells (CHO) and mouse keratinocyte (Pam). The accumulation of GFP-CDTB-(48–124) was also observed in the nucleus of these cells (not shown).

CDTB Is Composed of an N-terminal Domain for Nuclear Transport and a C-terminal Activity Domain—Comparison of amino acid sequences of CDTB component from a variety of

bacteria with members of the DNase-I family revealed that there are five conserved regions implicated as essential amino acid residues for DNase activity, as shown in Fig. 3D (boxed). Among them, all but Glu⁶⁶ are located in the C-terminal half of the CDTB molecule. Previously, Elwell and Dreyfus demonstrated that His¹⁵⁴, Asp²²⁹, Asp²⁶⁰, and His²⁶¹ of *E. coli* CDTB are essential for the activity, but Glu⁶⁶ is not (14). Taken together with the notion that N-terminal 48–124 aa plays a role as a domain for nuclear transport, we hypothesized that CDTB forms a two-domain structure composed of the N-terminal domain and C-terminal genotoxic activity domain. Therefore, we tried to swap the N-terminal 48–124 aa with SV40 T NLS, a classical monopartite NLS, and constructed a plasmid encoding a His-CDTB-(23–47 aa)-SV40 T NLS-CDTB-(125–283 aa) chimeric protein (Fig. 4A, CDTB Δ 48–124 aa-SV40 T NLS). The chimeric protein was purified to homogeneity (Fig. 4B) and microinjected into HeLa cells. After microinjection, immunostaining of the CDTB Δ 48–124 aa-SV40 T NLS showed it was imported into the nucleus of the HeLa cells as expected (Fig. 4C). Concomitantly, the nuclei exhibited a marked disappearance of chromatin in 4 h, which was very similar to that induced by microinjection of the wild type CDTB (Fig. 1A). These results suggested that the CDTB Δ 48–124 aa-SV40 T NLS acted as a genotoxin, supporting our hypothesis that CDTB is composed of two domains, the N-terminal domain for nuclear transport and the C-terminal for genotoxic activity. Therefore we tried to see whether a holotoxin containing SV40

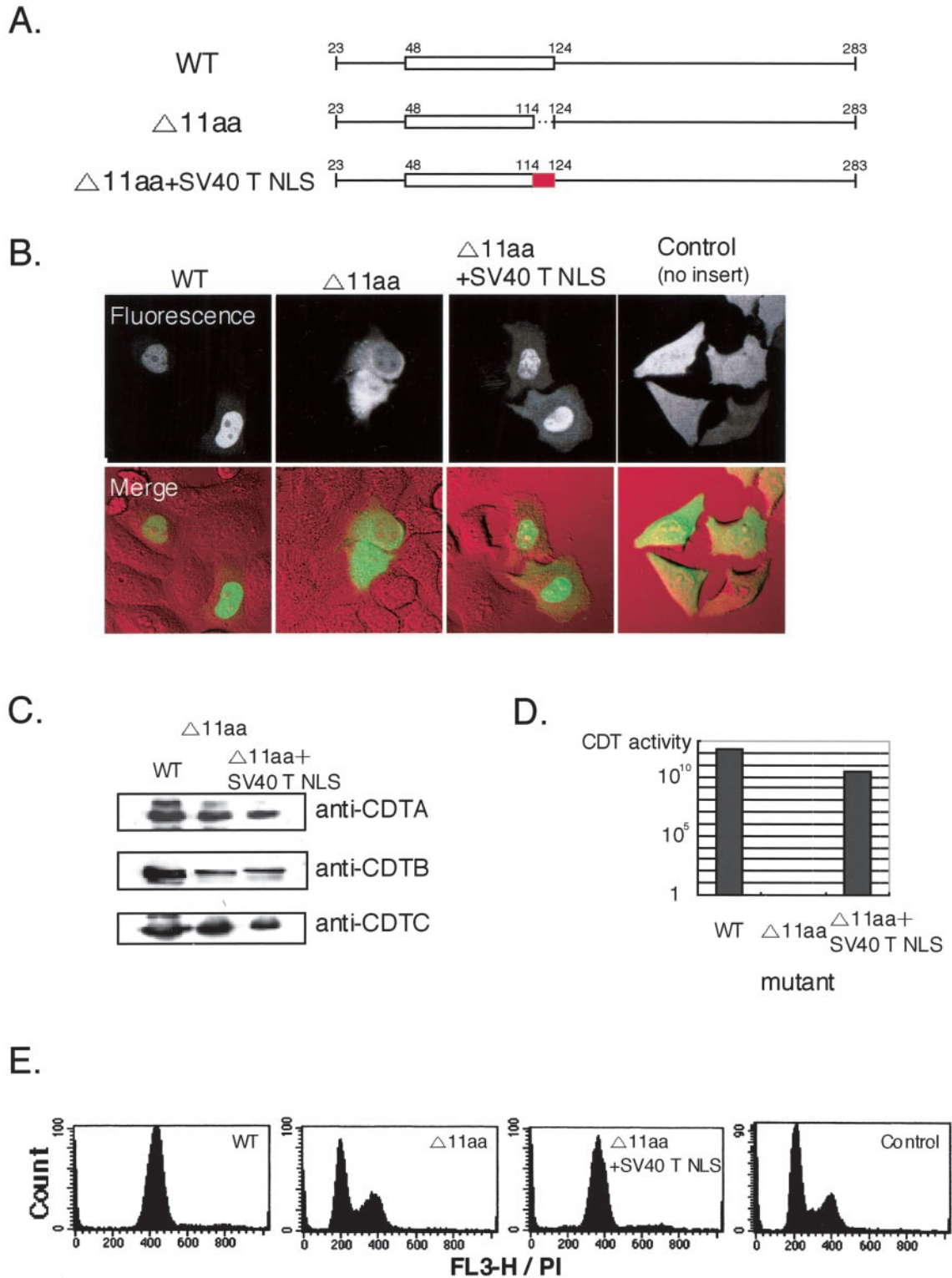


FIG. 5. Activity of holotoxin containing mutant CDTB with the 11-amino acid truncation in the NLS. *A*, schematic representation of wild type CDTB (*WT*), CDTB with truncation of 11 amino acids in position 114–124 (CDTB Δ 11aa (Δ 11aa)), and CDTB Δ 11aa in which truncation was replaced with SV40 T NLS (Δ 11aa + SV40 T NLS). *B*, transient expression of mutant CDTB and its localization. Wild type CDTB, CDTB Δ 11aa, and CDTB Δ 11aa-SV40 T NLS were expressed transiently by pEGFP-constructs in HeLa cells as GFP fusion protein. The GFP fusion protein was monitored by confocal microscopy using the FITC channel signal (*Fluorescence*). Computer-generated overlays of the fields in the fluorescence (*green*) and those of phase contrast microscopy (*red*) are shown (*Merge*). *C*, Western blotting analysis of purified CDT holotoxin of wild type, holotoxin containing CDTB Δ 11aa, and holotoxin containing CDTB Δ 11aa-SV40 T NLS. Holotoxin was purified by His tag sequence fused to the C terminus of CDTC using a Ni-NTA column. Western blotting analysis was performed using anti-CDTA, anti-CDTB, or anti-CDTC as the primary antibody. *D*, cytodistending activity of CDT holotoxin of wild type, holotoxin containing CDTB Δ 11aa, and holotoxin containing CDTB Δ 11aa-SV40 T NLS. One microgram of the purified holotoxin was incubated with a 10-ml culture of HeLa cells for 24 h. CDT activity was estimated as 50% cytotoxic dose, which was titrated as the endpoint of the highest 2-fold dilution of the sample showing 50% cytodistending cells after 72 h of incubation; CDT activity was defined as the reciprocal of the dilution. *E*, flow cytometry analysis of HeLa cells treated with CDT holotoxin of wild type, holotoxin containing CDTB Δ 11aa, and holotoxin containing CDTB Δ 11aa-SV40 T NLS. One microgram of the purified holotoxin was incubated with a 10-ml culture of HeLa cells for 24 h. *Control*, cells without treatment.

T NLS-CDTB chimera retains cytolethal distending activity. However, the experiment to purify the holotoxin containing CDTB Δ 48–124 aa-SV40 T NLS, CDTA, and CDTC with the C-terminal His tag indicated that they do not form a complex. This is probably because a drastic structural alteration of the CDTB prevented the formation of a complex with CDTA and CDTC. Knowing that CDTB(48–124) is the minimum region required for nuclear transport, we designed two CDTB mutants, one with an 11-amino acid truncation in region 114–124 (CDTB Δ 11aa) and the other with the 11 amino acids truncation replaced with SV40 T NLS (CDTB Δ 11aa-SV40 T NLS) (Fig. 5A). When CDTB Δ 11aa was transiently expressed as a GFP fusion protein in HeLa cells, the GFP fusion protein was ubiquitously found in the cell cytoplasm indicating that CDTB Δ 11aa had lost its ability to localize in the nucleus (Fig. 5B). On the other hand, GFP-CDTB Δ 11aa-SV40 T NLS expressed in HeLa cells was found to localize in the nucleus. CDT holotoxins containing the respective chimeric CDTB were successfully purified from the periplasmic fraction of recombinant *E. coli* using His tag in the C terminus of CDTC (Fig. 5C). Wild type holotoxin (*WT*) revealed strong cytodistending activity and successfully arrested the HeLa cells at G₂/M (Fig. 5, D and E). On the other hand, CDT holotoxin containing CDTB Δ 11aa (Δ 11aa) completely lost its activity for cytodistension and cell cycle block, whereas CDT holotoxin containing CDTB Δ 11aa-SV40 T NLS (Δ 11aa+SV40 T NLS) restored those activities. Taken together, these results strongly suggest that entry of CDTB into the nucleus is necessary for the induction of cytopathic effect by CDT holotoxin.

DISCUSSION

CDT is the first bacterial protein toxin found to be seemingly active as a DNase in target mammalian cells (14, 15). Previous studies suggest that CDTB is a toxic component and that CDTA and -C are carriers of CDTB to target cells (15, 28). Cortes-Bratti *et al.* (29) demonstrated that *H. ducreyi* CDT was internalized and transported to the Golgi using clathrin-coated pits (29). The final destination of CDTB is suggested to be the nucleus (15). As demonstrated in previous work (15) and in this study, transiently expressed CDTB or microinjected CDTB are transported into the nucleus. We have demonstrated that CDTB is transferred to the HeLa cell nucleus by active transport and that CDTB possesses a unique functional domain for nuclear transport. This is the first demonstration that a bacterial toxin is transferred to the animal cell nucleus by active transport.

The nuclear transport of bacterial component was first demonstrated by *Agrobacterium tumefaciens* virulence-related proteins (30). *A. tumefaciens* interacts with plant cells and is able to transfer DNA to the plant cell (31). The bacterial DNA, called T-DNA, which is a portion of the tumor-inducing plasmid of *Agrobacterium*, travels from *Agrobacterium* into the plant cells where the T-DNA integrates into the plant cell nuclear genome. For this purpose, the T-DNA must traverse the cytoplasm into the nucleus. This movement is carried out by two virulence-related proteins bound to T-DNA, called VirD2 and VirE2 (32, 33). These proteins have been demonstrated to possess a bipartite NLS (34, 35). Another example is the protein AvrBs3 produced by *Xanthomonas campestris* pv. *Vesicatoria* (36). In pepper plants, AvrBs3 is targeted to host plant cells by the bacterial Hrp type III secretion system and is transported into the nucleus by a monopartite NLS in the C terminus of the protein (37). It induces a rapid, localized cell death of the target plant. In the case of CDTB, a homology search or domain matching of CDTB amino acid sequences using available data bases did not hit any of the NLS candidate sequences. To investigate the region involved in the nuclear transport in the CDTB amino acid sequences, a series of deletion mutants of

CDTB fused to GFP were constructed, and localization of the GFP fusion protein was monitored. In some cases, the intensity of fluorescence appeared equal in both the nucleus and the cytoplasm. This applied to constructs 23–93, 23–59, and 23–102. These constructs appeared to retain partial activity to transport the GFP fusion protein into the nucleus. Finally the domain from the 48th to 124th aa was found to be the shortest stretch. Alignment of the CDTB amino acid sequences of a variety of bacteria clearly indicated that the amino acid sequences in residues 48–124 were highly conserved among CDTBs (Fig. 3C). However, there are no basic amino acid clusters, which are often observed in classical NLSs of nuclear proteins, in this region.

In the case of eukaryotic or viral nuclear proteins, classical NLSs require binding proteins that act as receptors to carry the NLS to the NPC and to pass through the pore (38–40). In *Saccharomyces cerevisiae*, 14 receptors have so far been found by sequence homology, and mammalian cells are supposed to possess more receptors, including importins (21, 41). Our preliminary attempt to see the interaction between CDTB and importin- α or - β failed to show any direct association. The *in vitro* transport assay demonstrated that nuclear transport of His-CDTB-GFP required the HeLa cell cytosol and an energy source, suggesting that CDTB might use unknown factor(s) to enter into the nucleus.

The chromatin collapse induced by microinjection of His-CDTB or FLAG-CDTB could not be observed in CDT holotoxin-intoxicated cultured cells. Lara-Tejero and Galan (15) suggested that this difference was because of the difference of the intracellular concentration of CDTB molecule, and they demonstrated cytodistension by microinjecting a very low concentration of *C. jejuni* CDTB into the cytosol. Elwell and Dreyfus (14), and Lara-Tejero and Galan (15) independently identified that CDTB has significant similarity to the DNase I family. The essential amino acids for metal binding and catalytic domains of the DNase I family are conserved in the CDTB family, and all of them are located at the C terminus of CDTB (14). Site-directed mutations of these amino acids in CDTB resulted in inactivation of chromatin-disintegrating activity when microinjected. In this study, we have hypothesized that CDTB forms a modular structure composed of an N-terminal domain for nuclear transport and a C-terminal DNase-like domain. We could also reproduce chromatin collapse by microinjecting CDTB with its N-terminal domain for nuclear transport exchanged with SV40 T NLS. These results further support the hypothesis that CDTB acts as a DNase-like molecule and that the N-terminal domain may not be important *per se* for genotoxic activity. Furthermore, we demonstrated that nuclear entry of CDTB is necessary for cytodistension and the cell cycle block by CDT holotoxin. Recently, two groups reported that CDT induced ATM-dependent early response, which is observed by DNA strand breaks induced by irradiation. Li *et al.* (42) demonstrated that CDT induces phosphorylation of H2AX and Mre11 relocalization in HeLa cells. Another study demonstrated that CDT induces the formation of Rad50 foci in primary human fibroblast (43). These results further suggest that CDT directly induces DNA damage. However, the discrepancies between the reported specific nuclease activities of CDTB and those of DNase I remain an open question. The precise molecular mechanism by which CDTB induces DNA damage remains to be elucidated.

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**An N-terminal Segment of the Active Component of the Bacterial Genotoxin
Cytotoxic Distending Toxin B (CDTB) Directs CDTB into the Nucleus**
Shuichi Nishikubo, Masaru Ohara, Yoko Ueno, Masae Ikura, Hidemi Kurihara, Hitoshi
Komatsuzawa, Eric Oswald and Motoyuki Sugai

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