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Localization of Diphtheria Toxin Nuclease Activity to Fragment A

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We describe a series of experiments that aimed to establish whether nuclease activity is actually associated with diphtheria toxin (DTx) and its A subunit (DTA), as we originally reported (M. P. Chang, R. L. Baldwin, C. Bruce, and B. J. Wisnieski, Science 246:1165–1168, 1989). Here we show that (i) trypsinization of DTx does indeed produce nucleolytically active DTA, (ii) reduction of electroeluted, unreduced, cleaved DTx (58 kDa) yields nuclease-active DTA (24 kDa), and (iii) fractionation of DTx and DTA by anion-exchange chromatography leads to coelution of nuclease activity with both forms of the toxin, even though each form elutes at a distinct salt concentration. In addition, we show that *Escherichia coli*-derived DTA also expresses nuclease activity. These studies confirm our initial assertion that the nuclease activity observed in DTx preparations is intrinsic to the DTA portion of DTx.

Diphtheria toxin (DTx) is synthesized as a single polypeptide chain that can be "nicked" by limited proteolysis to generate A and B subunits (DTA and DTB, respectively) that remain connected by a cystine bridge (7, 14, 16). DTB is involved in receptor binding; following sequestration in endosomes and subsequent acidification of the endosomal compartment, DTA is transported to the cytosol (8, 14, 22). DTA is involved in the ADP ribosylation of elongation factor 2, which leads to the inhibition of protein synthesis (7, 14, 16). We and others have noted that extensive toxin-induced translation inhibition is not correlated with cell death in human K562 cells (4, 19); these cells are lysis resistant to doses of DTx as high as 7.5 μg/ml despite the rapid and complete abrogation of protein synthesis (4). In addition, extensive inhibition of protein synthesis by unrelated treatments does not lead to the prelytic internucleosomal DNA cleavage that we have observed in DTx-intoxicated cells or to cell lysis (4). These and other findings (5) led us to propose that DTx triggers the programmed cell death pathway (4). Testing this hypothesis resulted in the discovery of a nuclease activity intrinsic to the DTx molecule (3). Detection of this activity is highly reproducible (10), optimal reaction conditions have been established (13), and an ADP ribosyltransferase (ADPrT)-defective form of DTx (called CRM197) exhibits greater nuclease activity than DTx itself (2). Moreover, nuclease activity was found to comigrate with the DTA portions of both DTx and CRM197 during electrophoresis in DNA-containing sodium dodecyl sulfate (SDS) gels (2, 3, 10) and with whole DTx and CRM197 during native gel electrophoresis (2), even though each of these forms of DTx migrates with a distinctive mobility in each gel system. We now report that the nuclease activity exhibited by DTx is intrinsic to DTA.

Proteolytic digestion of DTx. Recently, Wilson et al. reported that they were able to separate a nuclease-active fraction from an ADPrT-active fraction by anion-exchange chromatography of DTx and trypsin-generated DTA (21); therefore, they proposed that the nuclease activity of their DTx preparations resides in an uncharacterized contaminating protein. Moreover, they showed that trypsin cleavage of intact DTx results in a severe decrease in total observed nuclease activity and a simultaneous increase in ADPrT activity (21). In contrast, we observed that endoproteinase ArgC-cleaved DTx remains fully active and that the amount of DTx-associated nuclease activity corresponds to the amount of DTA generated (2, 3, 10). Here, we show that when monomeric DTx (3) is digested with tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Worthington) under mild conditions (i.e., at a 1:1,000 [wt/wt] ratio of trypsin to DTx, 1 μg of DTx per μl, as described by Williams et al. [20], rather than at a 10:1 ratio as employed by Wilson et al.), there is a concomitant increase in DTA and comigrating nuclease activity (Fig. 1A). Whole DTx (58 kDa) is unable to renature efficiently after being boiled in SDS, and hence it rarely exhibits nuclease activity in this assay (3, 10). Furthermore, Fig. 1A shows that the subsequent decrease in DTA-associated nuclease activity corresponds to proteolytic degradation of DTA to a 20-kDa peptide. Electrophoresis of the 3- and 18-h samples under nonreducing conditions (Fig. 1A) demonstrates that the disappearance of the 20-kDa peptide correlates with the appearance of a new band at ~54 kDa, which represents the 20-kDa fragment of DTA attached to DTB by the cystine bridge (Cys-186–Cys-201). Amino acid sequencing revealed that the 20-kDa fragment is the DTA fragment created by trypsin removal of 39 residues from the amino terminus (23). Because the 20-kDa fragment does not express nuclease activity in this assay, it appears that some or all of the amino terminus is required for nuclease activity.

To rule out the possibility that DTx preparations are contaminated with a nuclease with the same M_r as DTA (i.e., ~24,000), ArgC-cleaved DTx (250 μg [3]; List Biological Laboratories, Inc.) was electrophoresed under nonreducing conditions in a 1-h prerun SDS-polyacrylamide gel. DTx (58 kDa) was localized and electroeluted into 1 ml of 0.01% SDS–2.5 mM Tris–20 mM glycine, pH 8.3. A portion (50 μl) was solubilized in reducing buffer and electrophoresed in an

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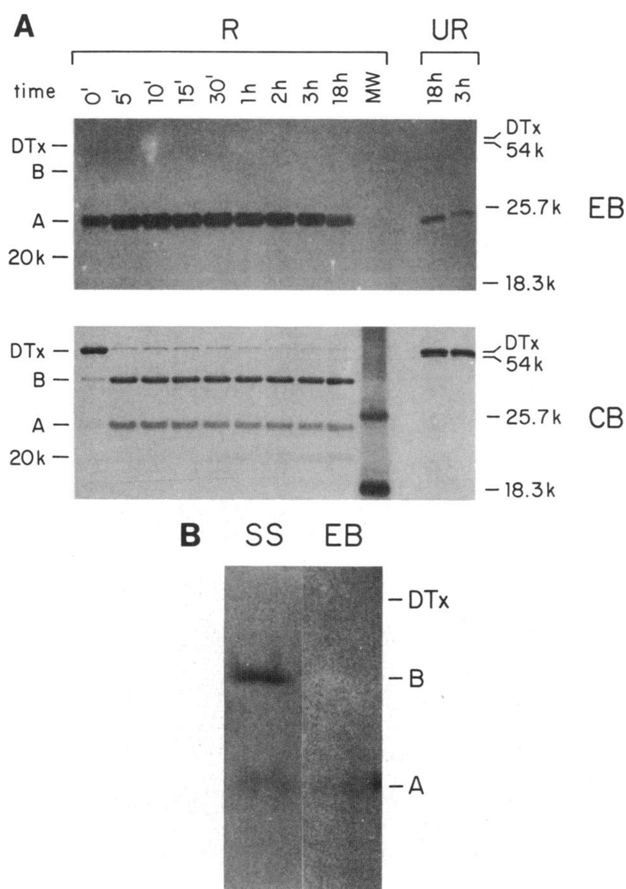

FIG. 1. Levels of DTA correspond to levels of nuclease activity. (A) The effect of mild trypsin cleavage on DTA nuclease activity. After trypsin treatment of DTx for the indicated times (20), samples were electrophoresed in an SDS-DNA gel and monitored for nuclease activity (3). R and UR denote reduced and unreduced samples. Gel EB is ethidium bromide stained; gel CB is the same gel after Coomassie blue staining. DTB and DTA are labeled B and A; lane MW contains molecular weight markers. Molecular masses are given in kilodaltons. (B) The nuclease-active species is a 58-kDa protein before reduction and a 24-kDa protein after reduction. EB indicates ethidium bromide staining; SS indicates the same gel after silver staining. The strong spot of nuclease activity at the right edge of DTA is in this lane because there were no neighboring samples.

SDS-DNA gel (3). The results (Fig. 1B) indicate that the nuclease activity is indeed intrinsic to the DTA domain of DTx, as it copurifies with a 58-kDa species under nonreducing conditions and yields a 24-kDa nuclease-active species following chemical reduction. Also apparent in Fig. 1B is a band of increased fluorescence corresponding to DTB, indicating DNA binding by the B subunit. This phenomenon is observed frequently. It should be noted that gels that are stained for protein after incubation for expression of nuclease activity show slightly lower ratios of DTA to DTB because DTA renatures very effectively and leaches out of the gel matrix. DTB aggregates upon detergent removal, and hence very little leaching occurs.

Anion-exchange chromatography of DTx. Our next objective was to establish whether the nuclease activity observed in our nominally pure preparations of uncleaved DTx and ArgC-nicked DTx would remain associated with DTx and DTA during fast performance liquid chromatography (FPLC) as conducted by Wilson et al. (21). Uncleaved DTx (100 μg; List) in 1 ml of buffer A (10 mM Tris [pH 7.6], 0.2 mM EDTA) was chromatographed on a Mono-Q HR 5/5 anion-exchange FPLC column (5- by 50-mm bed volume; Pharmacia) with a linear gradient of 0 to 200 mM NaCl (21, 10). Fractions (1 ml) were immediately subdivided and stored at −85°C. Figure 2A shows the elution profile. Nuclease activity towards λ phage DNA was detected in fractions 37 through 40 (peak centered on fraction 38; Fig. 2B). No nuclease activity was detected in any early-eluting fractions, in distinct contrast to the reported findings of Wilson et al. (21). It should be noted that typical commercial preparations of "intact" DTx are ~5% cleaved (primarily into DTA and DTB subunits). Under the nonreducing, nondenaturing FPLC conditions employed in Fig. 2A, the bulk of DTA and DTB should remain associated. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of concentrated fractions 1 to 62 revealed that only fractions 37 to 42 contained protein (DTx). In Fig. 2C, fractions 37, 39, and 41 were run uncleaved, and fractions 38, 40, and 42 were nicked with endoproteinase ArgC before SDS-PAGE under reducing conditions. The presence of cleavage fragments DTA and DTB demonstrates that the material eluting in these six fractions is indeed DTx. The smaller peak of toxin protein (at fraction 41) may consist of dimeric DTx, which is nucleolytically inactive (6, 10).

Anion-exchange chromatography of DTA. To substantiate the nuclease activity of DTA, ArgC-cleaved DTx (150 μg each; List and Calbiochem Corporation) was run reduced on a prerun (1 h, 180 V) SDS–12.5% polyacrylamide gel. DTA was localized and electroeluted as in Fig. 1B. β-Mercaptoethanol (β-ME) was added to 10 mM, and the pooled sample was dialyzed (Spectrapor 3; 3.5-kDa cutoff, 33 h, 4°C) against two changes (1 liter each) of buffer A (Fig. 2A) that contained 10 mM β-ME. DTA was then further purified by chromatography on a virgin Mono-Q column (Fig. 3A). DTA appeared centered on fractions 33/34 and 38 (Fig. 3B); this was confirmed by the presence of ADPrT activity (Fig. 3A). Fractions 33 and 34 exhibited strong nuclease activity (Fig. 3C). Fractions 37 to 39 showed weaker activity and had much less protein (Fig. 3C). Therefore, we fractionated two species of DTA that may differ in charge or conformation or both. It should be noted that three forms of DTA can be generated by ArgC treatment, with either one (Arg-190), two (Arg-190 and Arg-192), or three arginines (Arg-190, Arg-192, and Arg-193) at the carboxy terminus. These three forms (designated A′, A″, and A‴) could presumably be fractionated because of charge differences. Indeed, isoelectric focusing (IEF) (Fig. 3D) revealed that A‴ was the predominant component of fractions 33 and 34, whereas A″ was the predominant component of fraction 38. The IEF results confirm that the 24-kDa protein seen in both nuclease-active peaks is indeed DTA.

Separation of DTA and DTB by anion-exchange chromatography. A second method of confirming the nuclease activity of DTA involved FPLC of ArgC-cleaved DTx under reducing, denaturing conditions. Here, toxin reduction and denaturation with urea would be expected to release any spuriously bound contaminants. ArgC-cleaved DTx (200 μg; Calbiochem) in 1 ml of 10 mM Tris (pH 7.6)–8 M urea–120 mM dithiothreitol–0.2 mM EDTA was chromatographed on a Mono-Q column (linear 0 to 200 mM NaCl gradient; 1-ml fractions). The urea elution buffer contained 10 mM β-ME instead of 120 mM dithiothreitol. The FPLC elution profile is shown in Fig. 4A. After dialysis to remove urea, ADPrT activity was associated with fractions 25, 26, and 28 (Fig.

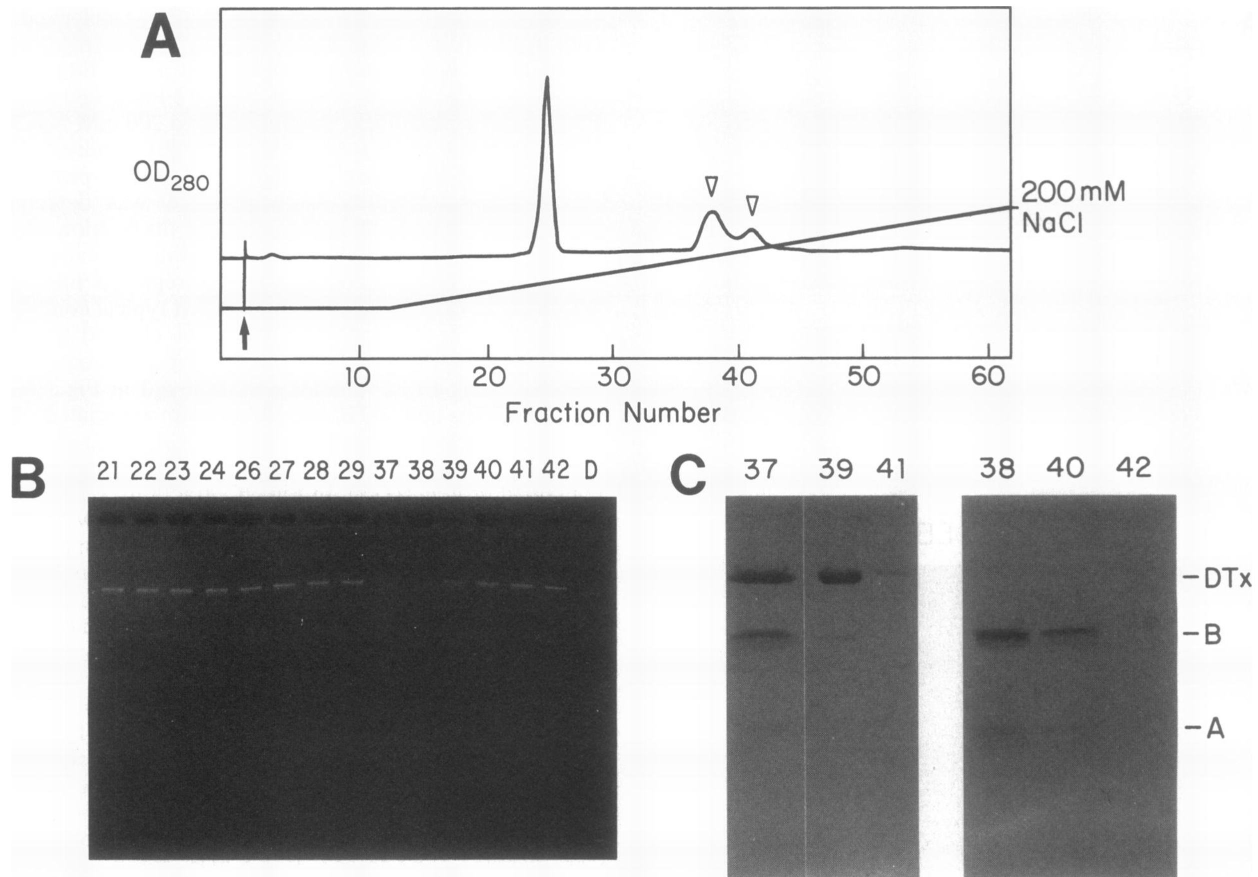

FIG. 2. Nuclease activity copurifies with DTx after anion-exchange chromatography. (A) The OD_{280} (optical density at 280 nm) peak at fraction 24 is attributed to EDTA because it was present in control elutions conducted without protein (17). The two peaks marked by open arrowheads contain DTx (see panel C). (B) Nuclease activity was localized by concentrating fractions (0.8 ml of each) to 50 μl on Centricon-10 microconcentrators. After adding 50 μl of Tris (10 mM, pH 7.6), 10 μl of each fraction was assayed for nuclease activity in 40 μl (final volume) of 10 mM Tris (pH 7.6)–2 mM $CaCl_2$–2 mM $MgCl_2$–1 mM $MnCl_2$ containing 0.2 μg of phage λ DNA (New England Biolabs). The reaction mixtures were incubated for 4 h at 37°C before electrophoresis (10 μl applied per lane) on a 1% agarose gel (3). Sample D contained 0.2 μg of DTx starting material. (C) SDS-PAGE of concentrated FPLC fractions. Fractions 37, 39, and 41 were run uncleaved; fractions 38, 40, and 42 were nicked with ArgC before SDS-PAGE under reducing conditions (3, 10). DTx, B, and A show the positions of whole DTx, DTB, and DTA after staining with Coomassie blue.

4A). These same fractions also exhibited nuclease activity in a λ DNA digestion assay (Fig. 4B). Fraction 27 exhibited nuclease activity as well, but at a much lower level. Figure 4C shows that fraction 25 contains DTA, fractions 26 and 27 contain DTB and DTA, and fraction 28 contains some uncleaved DTx, DTB, and DTA. As uncleaved DTx is not an active form of ADPrT, its presence in fraction 28 explains the lower ADPrT level of this fraction. In Fig. 4C, we also demonstrate that the nuclease activity in fractions 25 to 28 comigrates with the DTA subunit during SDS-PAGE in a DNA-embedded gel. Thus, the dark holes in the ethidium bromide-stained gel correspond to DTA bands visualized by Coomassie blue staining. Because whole DTx (58 kDa) rarely expresses nuclease activity in this assay, the amount of nuclease activity observed corresponds to the amount of free DTA. The fact that the shapes of nuclease-active bands conform exactly to the shapes of DTA protein bands is in itself strong evidence of their being identical. Quantitative recovery of nuclease activity is demonstrated in Fig. 4C (compare lane C with lane 25), where the levels of nuclease activity in the DTA band of unfractionated ArgC-cleaved DTx (i.e., starting material) and FPLC-purified DTA are very similar. Furthermore, the nuclease and ADPrT activities of fraction 25 are both higher than those of fraction 26 (Fig. 4). Any losses that we have encountered in nuclease activity (e.g., with repeated freeze-thawing) have always been correlated with losses in ADPrT activity. The same set of fractions are also shown in a duplicate half of the SDS-DNA gel that was fixed and silver stained immediately after electrophoresis (i.e., the gel was neither washed nor incubated for in situ expression of nuclease activity). To confirm that these samples truly contain DTA, IEF was conducted. The three characteristic DTA bands shown for fraction 26 (Fig. 4C, IEF) were seen exclusively in fractions 25 to 28. However, fraction 25 contains almost exclusively A‴, whereas fraction 26 contains predominantly A‴ and A″ in equal amounts (as shown). The higher ADPrT and nuclease activities of fraction 25 suggest a higher renaturation efficiency or higher intrinsic activity levels of the A‴ form of DTA. The selective loss of DTA from fraction 25 from the SDS-DNA gel suggests that A‴ is more effective at renaturation (and hence leaching from the gel).

Comigration of nuclease activity with *Escherichia coli*-derived DTA. Figure 5A shows that samples of FPLC-

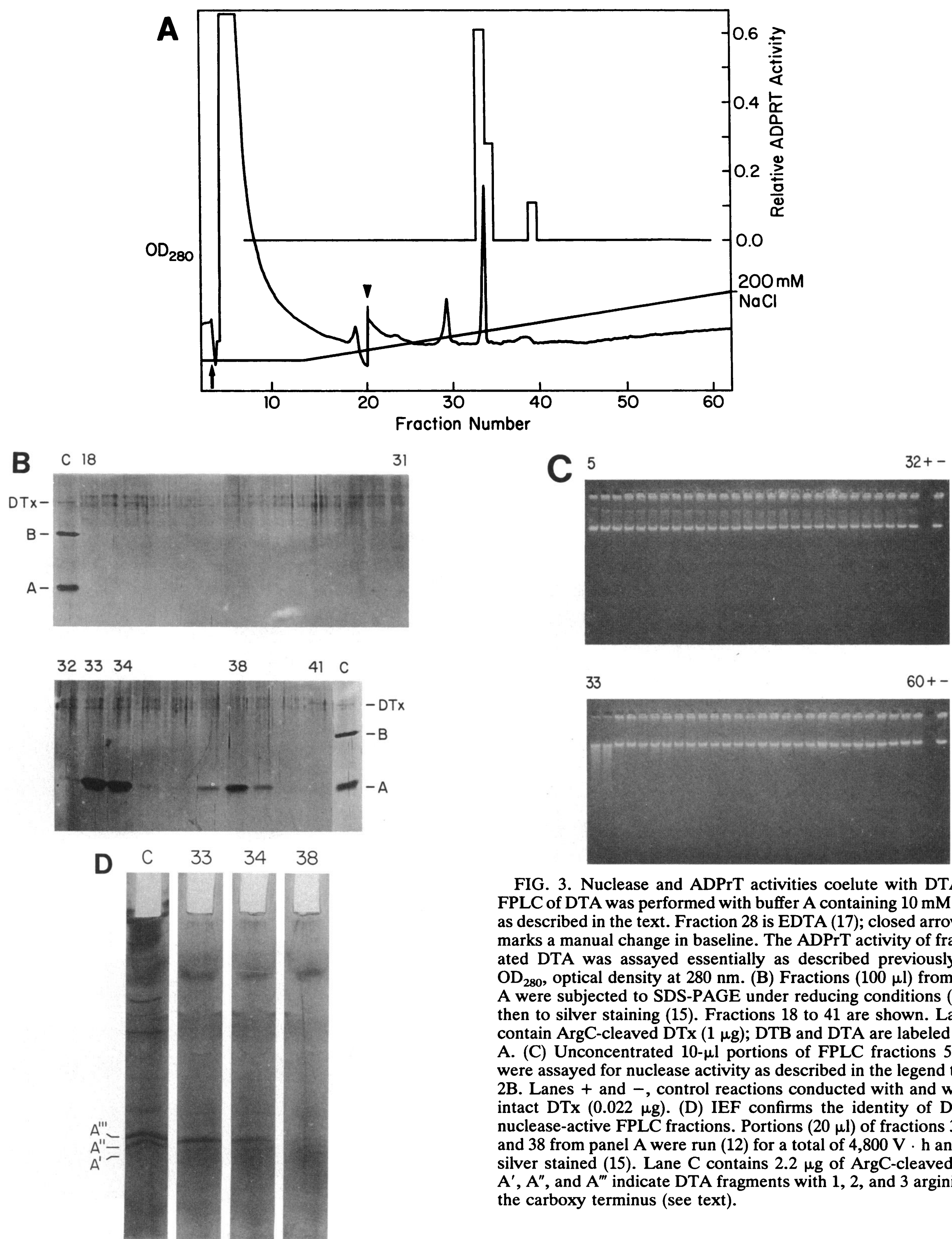

FIG. 3. Nuclease and ADPrT activities coelute with DTA. (A) FPLC of DTA was performed with buffer A containing 10 mM β-ME as described in the text. Fraction 28 is EDTA (17); closed arrowhead marks a manual change in baseline. The ADPrT activity of fractionated DTA was assayed essentially as described previously (21). OD_{280}, optical density at 280 nm. (B) Fractions (100 μl) from panel A were subjected to SDS-PAGE under reducing conditions (9) and then to silver staining (15). Fractions 18 to 41 are shown. Lanes C contain ArgC-cleaved DTx (1 μg); DTB and DTA are labeled B and A. (C) Unconcentrated 10-μl portions of FPLC fractions 5 to 60 were assayed for nuclease activity as described in the legend to Fig. 2B. Lanes + and −, control reactions conducted with and without intact DTx (0.022 μg). (D) IEF confirms the identity of DTA in nuclease-active FPLC fractions. Portions (20 μl) of fractions 33, 34, and 38 from panel A were run (12) for a total of 4,800 V · h and then silver stained (15). Lane C contains 2.2 μg of ArgC-cleaved DTx; A′, A″, and A‴ indicate DTA fragments with 1, 2, and 3 arginines at the carboxy terminus (see text).

purified DTA and DTx fractions obtained from B. A. Wilson and R. J. Collier (21) also contain a 24-kDa nuclease-active band (lanes 1 and 2). Indeed, eight of nine toxin samples obtained from this group exhibited nuclease activity; the only exception was the FPLC-purified DTA sample described in reference 21, which, interestingly, contained no protein by silver staining (data available upon request). Thus, it appears that our particular assay methods are reliably more sensitive at nuclease detection. Because it is highly unlikely that *E. coli* would secrete a nuclease identical

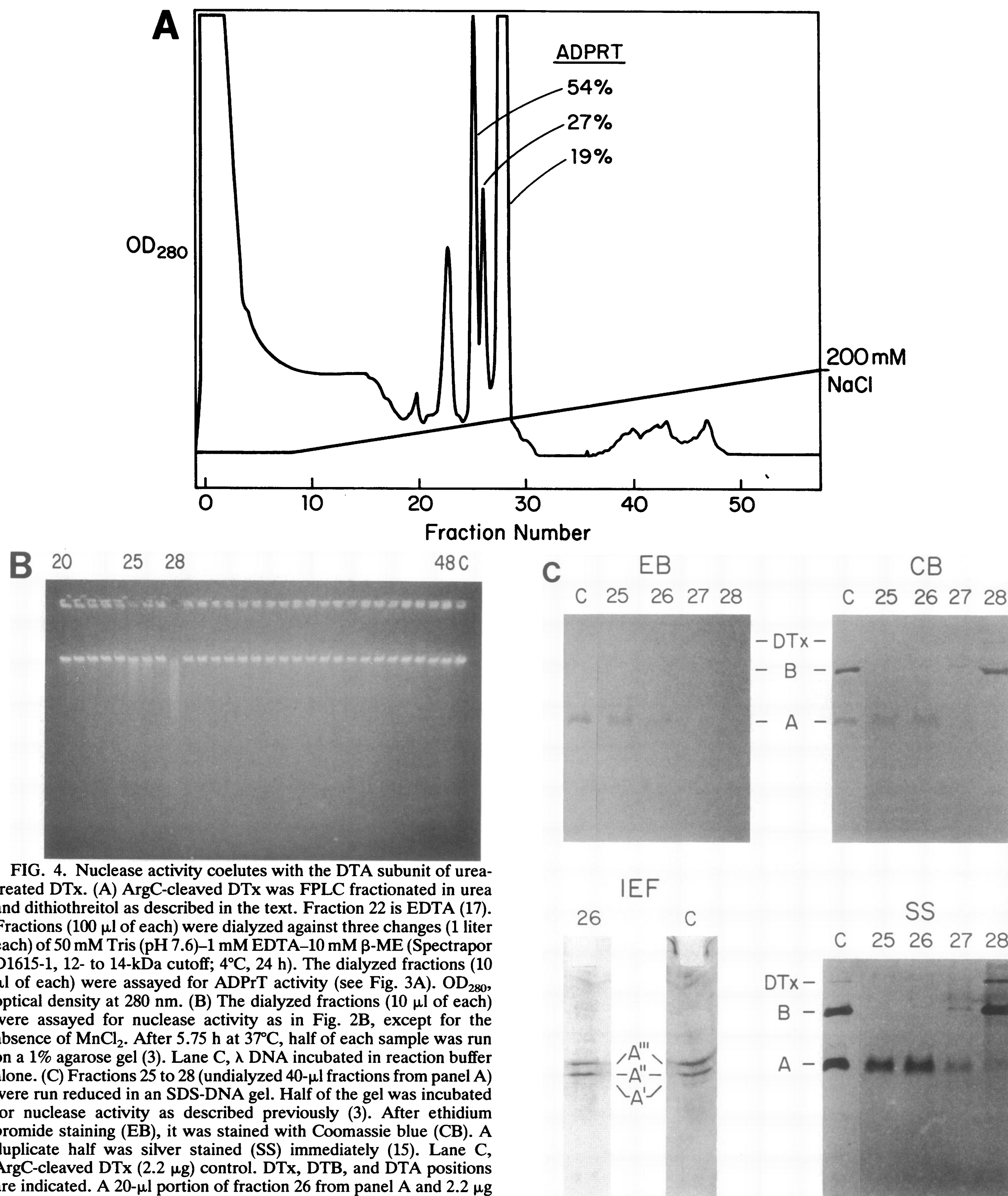

FIG. 4. Nuclease activity coelutes with the DTA subunit of urea-treated DTx. (A) ArgC-cleaved DTx was FPLC fractionated in urea and dithiothreitol as described in the text. Fraction 22 is EDTA (17). Fractions (100 μl of each) were dialyzed against three changes (1 liter each) of 50 mM Tris (pH 7.6)–1 mM EDTA–10 mM β-ME (Spectrapor D1615-1, 12- to 14-kDa cutoff; 4°C, 24 h). The dialyzed fractions (10 μl of each) were assayed for ADPrT activity (see Fig. 3A). OD_{280}, optical density at 280 nm. (B) The dialyzed fractions (10 μl of each) were assayed for nuclease activity as in Fig. 2B, except for the absence of $MnCl_2$. After 5.75 h at 37°C, half of each sample was run on a 1% agarose gel (3). Lane C, λ DNA incubated in reaction buffer alone. (C) Fractions 25 to 28 (undialyzed 40-μl fractions from panel A) were run reduced in an SDS-DNA gel. Half of the gel was incubated for nuclease activity as described previously (3). After ethidium bromide staining (EB), it was stained with Coomassie blue (CB). A duplicate half was silver stained (SS) immediately (15). Lane C, ArgC-cleaved DTx (2.2 μg) control. DTx, DTB, and DTA positions are indicated. A 20-μl portion of fraction 26 from panel A and 2.2 μg of ArgC-cleaved DTx (lane C) after IEF are also shown (see Fig. 3D). A‴, A″, and A′ are defined in the legend to Fig. 3D.

to that associated with DTx and DTA derived from *Corynebacterium diphtheriae*, we have examined the nuclease activity of FPLC-purified recombinant DTA from *E. coli*. This material (~4.5 μg in 100 μl) was also a kind gift of B. A. Wilson and R. J. Collier and was used as supplied. Having established by SDS-PAGE and silver staining that it contained protein and that it was capable of digesting λ DNA in solution (data not shown), our next objective was to ascertain whether the nuclease activity was intrinsic to a 24-kDa protein. In Fig. 5B, we show that electrophoresis of 0.2 μg of this DTA preparation in an SDS-DNA gel yields two nu-

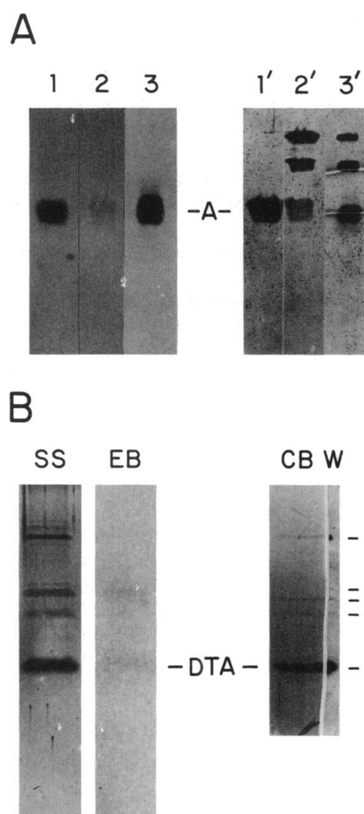

FIG. 5. FPLC-purified DTA and DTx from B. A. Wilson and R. J. Collier (21) express nuclease activity. (A) Samples were electrophoresed in an SDS-DNA gel (3). Lanes 1 to 3 are ethidium bromide stained. Lane 1, FPLC-purified DTA (3 μg); lane 2, FPLC-purified DTx (8.4 μg); lane 3, ArgC-cleaved DTx (3.5 μg, standard; List). A (between the panels) shows the position of DTA. After SDS-PAGE, lane 3 was removed and incubated separately. Lanes 1′ to 3′ are lanes 1 to 3 after silver staining. (B) FPLC-purified *E. coli*-derived DTA exhibits nuclease activity. Lane EB is the ethidium bromide-stained SDS-DNA gel lane (0.2 μg of DTA applied). Lane SS is the same gel lane after silver staining. Lanes CB and W are two pieces of a blot (Immobilon-P; Millipore) of a gel lane that contained 3 μg of the *E. coli* DTA. Piece CB was Coomassie blue stained, and the nuclease-active protein bands were sequenced. Piece W shows bands recognized by a monoclonal antibody against DTA.

clease-active bands. One migrates as a 24-kDa protein, and the other migrates as a higher-molecular-weight band. Amino acid sequencing of the 24-kDa nuclease-active band identified it as DTA with an N-terminal methionine (20 sequencing cycles). The other nuclease-active band contained insufficient protein (Fig. 5B, lane CB) for the determination of an unambiguous sequence. However, Western blot (immunoblot) analysis of a slice of the blotted gel lane that was used for amino acid sequencing established that both nuclease-active bands react with an anti-DTA monoclonal antibody that was generously donated by J. M. Rolf and L. Eidels (Fig. 5B, lane W). The two pieces of blot (CB and W) are from an SDS gel lane that contained 3 μg of DTA.

Conclusion. In this report, we present substantial evidence that nuclease activity is an intrinsic property of the DTA portion of the DTx molecule. Although the chromatography procedures that we employed were identical to those that Wilson et al. employed to isolate an early-eluting nuclease "contaminant" (21), no corresponding nuclease-active fraction was ever observed in any of our toxin preparations. Thus, coelution of DTA with nuclease activity, ADPrT activity, and a protein of appropriate molecular weight and isoelectric properties has been confirmed for FPLC-purified DTA that was originally isolated from an SDS gel and dialyzed (Fig. 3) and for DTA that was derived by FPLC of cleaved DTx in the presence of urea and reducing agent (Fig. 4). In addition, electroelution of cleaved DTx leads to comigration of nuclease activity with DTA after sample reduction (Fig. 1B). Importantly, we demonstrate that our protocol for cleaving DTx consistently leads to DTA-containing fractions that exhibit both nuclease activity and ADPrT activity after anion-exchange chromatography. Thus far, we have tested a number of different toxin samples (DTA, monomeric intact DTx purified from toxin obtained from Connaught Laboratories, reduced urea-denatured cleaved DTx, and intact DTx purchased from List and Calbiochem) and have used four different Mono-Q columns and three different FPLC systems, but we have never observed a nuclease activity eluting in a low-salt fraction corresponding to that of Wilson et al. (21). Moreover, our assays of DTx and DTA samples that were FPLC purified independently by Wilson et al., including *E. coli*-cloned DTA, confirm the comigration of nuclease activity with DTA during FPLC and subsequent SDS gel electrophoresis (e.g., see Fig. 5). Amino acid sequencing and monoclonal antibody studies, such as those we described in this report for *E. coli*-derived DTA, are currently being conducted on protease-treated samples of DTx, CRM197, and their respective A subunits to pinpoint the regions involved in DNA cleavage (11). Recently, it has been discovered that intracellular expression of the CRM197 A subunit leads to cell death in both *Drosophila* spp. and diploid yeasts at 18°C (1, 18). New data show that <200 molecules will kill a diploid yeast cell (18). The only activity ascribed to the A chain of CRM197 is nuclease activity (2). The results of these extensive investigations substantiate our original conclusion, namely, that nuclease activity is intrinsic to the DTA portion of the DTx molecule. Consequently, our proposal (3, 5) that DTx "acts as a double-edged sword, using apparently non-overlapping sites to effect translation inhibition and chromosomal cleavage," remains a viable model for further study.

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