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# Cloning, Sequence Determination, and Expression in Transfected Cells of the Coding Sequence for the *tox* 176 Attenuated Diphtheria Toxin A Chain

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**DNA including the coding sequence for the A chain of the mutant diphtheria toxin *tox* 176 was cloned. The cloned mature A-chain coding sequence showed a G-to-A transition at nucleotide 383 as the only difference from the wild-type sequence. This resulted in replacement of the glycine at position 128 by aspartic acid in the predicted amino acid sequence. A eucaryotic cell expression plasmid, pTH1-176, was constructed in which the *tox* 176 A-chain coding sequence was attached to a truncated metallothionein promoter. The toxicity of this construct, compared with that of the corresponding wild-type diphtheria toxin A-chain plasmid, pTH1, was assessed after transfection into the human 293 cell line by an indirect transient expression assay (I. H. Maxwell, F. Maxwell, and L. M. Glode, Cancer Res. 46:4660–4664, 1986). For the same effect, 15- to 30-fold more pTH1-176 than pTH1 was required, a result consistent with previous in vitro estimates of the diminished activity of the *tox* 176 A chain. Controlled expression of the cloned *tox* 176 A-chain coding sequence may provide a means of eliminating specific cell populations in an organism, for which purpose the wild-type diphtheria toxin A chain might prove too toxic.**

We are exploring the use of controlled expression of an introduced gene encoding a toxin as a means of killing specific cell types. This idea should find applications in developmental biology and, eventually, in cancer therapy (13). In principle, selective lethality should be achievable by linking the toxin gene with tissue-specific transcriptional regulatory elements. We have described initial experiments along these lines (13) which used the coding sequence for the diphtheria toxin A chain (DT-A). This enzyme, when introduced into cells, inactivates elongation factor 2, causing inhibition of protein synthesis and consequent cell death (14). Once inside a cell, DT-A is extremely toxic, and there is evidence that the introduction of a single molecule of this protein per cell is lethal (21). The use of a wild-type DT-A gene may therefore pose a significant problem in terms of controlling expression since essentially no basal expression could be tolerated in nontarget cells. To lessen this problem we isolated the coding sequence for an attenuated DT-A, *tox* 176, which arose from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis (19) and which has been shown to have markedly diminished enzyme activity in vitro and toxicity for mammalian cells (19, 21). In this paper, we report the cloning and sequencing of *tox* 176 A-chain DNA, showing a single nucleotide difference from the wild-type DNA (resulting in a single amino acid change). Sequence data for wild-type DT-A (5) and several inactive DT-A mutants (2, 8) have previously been reported. To our knowledge, this is the first report of the sequence of an attenuated DT-A mutant which retains substantial toxicity. We also report on the effect of transfecting line 293 cells (4) with an expression plasmid for the *tox* 176 coding sequence compared with a corresponding plasmid containing the wild-type sequence.

The *tox* 176 DT-A coding sequence (0.58 kilobases [kb]) was cloned in plasmid pBR322 within a 1.1-kb *Hin*dIII-*Cla*I DNA fragment from genomic DNA of *Corynebacterium diphtheriae* lysogenic for the *tox* 176 bacteriophage β mutant (19). Although induction of the lysogen and isolation of β phage DNA (15) would have provided an initial enrichment for the desired sequence, this was offset by the difficulty of growing the phage to high titer. In our hands, cloning from genomic DNA of the lysogen proved more straightforward. The *C. diphtheriae tox* 176 lysogen was grown and lysed after penicillin treatment (15), and chromosomal DNA was isolated. To detect the *tox* 176 sequence, restriction digests of the DNA were subjected to Southern hybridization (16) by using a probe derived from the wild-type DT-A coding sequence, subcloned from pDT201 (10). This analysis (data not shown) revealed a single hybridizing band of ca. 6 or 1.1 kb after restriction with *Hin*dIII or *Cla*I plus *Hin*dIII, respectively. The latter band corresponded to the fragment expected from the DT DNA sequence (5, 8), extending from a *Hin*dIII site in the promoter region, through the A-chain coding sequence, to a *Cla*I site early in the B-chain coding region. Once isolated, clones containing this DNA fragment could be handled without special containment precautions since the regions of the B chain responsible for adsorption and introduction of the A chain into mammalian cells (14) were absent (being encoded downstream from the *Cla*I site).

As a precaution against inadvertent cloning of additional B-chain DNA, we first performed a size fractionation of the restriction digest, which also enriched for the desired fragment. The *Hin*dIII plus *Cla*I digest of genomic DNA (40 μg) was electrophoresed in a 5% polyacrylamide gel, and DNA in a size range of ca. 0.8 to 1.5 kb was recovered. This DNA was cloned into pBR322 cut with *Hin*dIII plus *Cla*I by standard methods (11), and clones containing the DT-A coding sequence were identified by colony hybridization (6) by using the probe described above. The identity of positive clones (designated pBRtox176) was confirmed by restriction digestion of minilysates (7) and Southern hybridization. These cloning steps were performed in a negative-pressure

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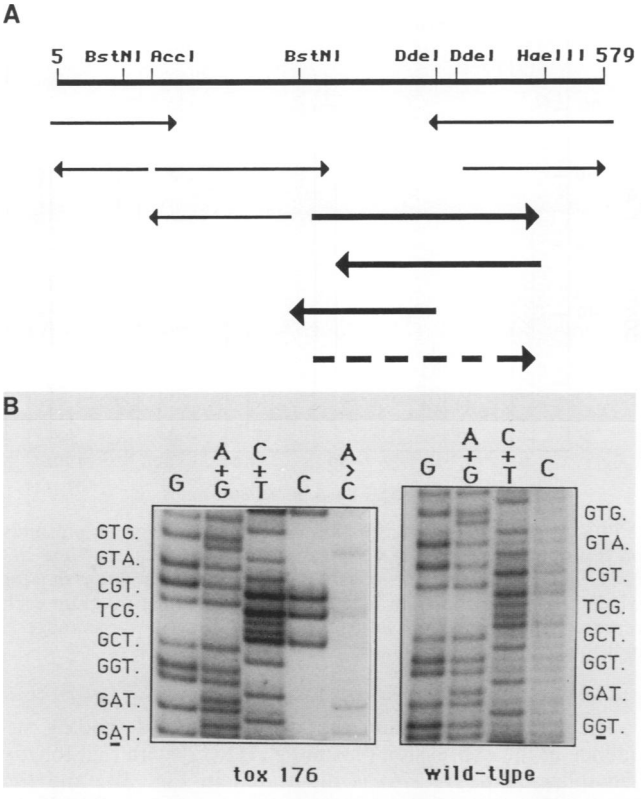


FIG. 1. (A) Sequencing strategy used for *tox* 176 DT-A coding sequence. The sequence from nucleotides 5 to 579 (end of DT-A) of the coding sequence for mature DT-A (5, 8) was determined. Nucleotides 2 through 4 were inferred from the presence of the *Hha*I site also present in the wild-type DT-A sequence. Thick arrows represent the three sequencing experiments in which the nucleotide change at position 383 was detected. The broken arrow represents a sequencing experiment using wild-type DT-A DNA, subcloned from pDT201 (10), in which the relevant part of the published sequence (5) was confirmed. (B) Sections of autoradiograms of sequencing gels of corresponding coding sequences determined for *tox* 176 and wild-type DT-A. Nucleotides 382 to 405 are compared, as determined from DNA fragments 5′ labeled at the *Bst*NI site (at nucleotide 258). The A and G at position 383 are underlined; this was the only difference observed between the two DT-A sequences. (C) Deduced amino acid sequence of *tox* 176 DT-A. The amino acid substitution observed in *tox* 176 (Asp for Gly at position 128) is boxed. The positions of amino acid substitutions in inactive DT-A mutants *tox* 197 (Glu for Gly at position 52) (2) and *tox* 228 (Asp for Gly at position 79 and Lys for Glu at position 162) (8) are underlined, as is the Glu at position 148 where deletion, or substitution of Asp, also results in activation, as shown by site-directed mutagenesis (18) (Emerick et al., DNA).

containment facility in accordance with National Institutes of Health guidelines. To facilitate sequencing, the *tox* 176 A-chain DNA was then subcloned as a 0.58-kb *Hha*I-*Sau*3AI fragment (*Hha*I end blunted with T4 DNA polymerase) into pUC19 (22), cut with *Hin*cII and *Bam*HI. This fragment included the DT-A sequence extending from the *Hha*I site in the first and second codons of the mature DT-A coding sequence to the *Sau*3AI site present exactly at the junction of the A- and B-chain sequences (5, 8).

Sequencing was performed on both strands of the cloned fragment by the procedure of Maxam and Gilbert (12), as described by Maniatis et al. (11), using restriction fragments labeled with $^{32}P$ at the 5′ termini; the sequencing strategy is indicated in Fig. 1A. The *tox* 176 A-chain sequence was found to differ from the wild-type sequence (5) by one nucleotide, a G-to-A transition at position 383 of the mature A-chain coding sequence. The relevant regions of autoradiograms of sequencing gels of wild-type and *tox* 176 DT-A coding sequences are shown in Fig. 1B.

The observed transition is in the second position of codon 128, resulting in the substitution of an aspartic acid for a glycine in the deduced amino acid sequence, as shown in Fig. 1C. Amino acid changes reported for several other (inactive) DT-A mutants are also indicated in Fig. 1C. The replacement of glycine by an acidic amino acid also occurs in two inactive DT-A mutants (glutamic acid at position 52 in *tox* 197 [2] and aspartic acid at position 79 in *tox* 228 [8]; a second replacement, lysine for glutamic acid at position 162, also occurs in *tox* 228). These observations suggest that the distribution of charged amino acids may be of particular importance in relation to DT-A function. It is also interesting that the change observed in *tox* 176 occurs only 20 amino acid residues from the glutamic acid at position 148, which forms part of the NAD-binding site, as shown by photoaffinity labeling (1). Deletion of the glutamic acid at position 148 (A. Emerick, L. Greenfield, and C. Gates, DNA **4**:78, 1985) or replacement by aspartic acid (18) results in loss of DT-A activity. Perhaps the presence of an additional negatively charged amino acid at position 128 in *tox* 176 DT-A may hinder NAD binding. Clearly, knowledge of sequence changes resulting in diminished DT-A activity will be of assistance in attempting to generate additional DT-A mutants with a range of toxicities by site-directed mutagenesis.

We have previously described construction of an expression plasmid, pTH1, for the wild-type DT-A coding sequence (13). This sequence was replaced by the corresponding *tox* 176 DT-A sequence to give pTH1-176. The structure of these plasmids is shown in Fig. 2A. The procedure used for substituting the *tox* 176 DT-A sequence was as follows. A 1.1-kb *Sau*3AI fragment from pBRtox176, containing the *tox* 176 DT-A sequence, was first subcloned into pSV2β-globin (17). DNA containing the mutant region of the *tox* 176 sequence (see above), together with downstream simian virus 40 mRNA processing signals (17), was excised with *Acc*I plus *Eco*RI and cloned between the corresponding restriction sites of pTH1. The resulting plasmid thus contained a chimeric DT-A sequence of which the first 102 nucleotides were derived from the wild-type sequence and the remaining 477 were derived from the *tox* 176 sequence. Since sequence analysis showed no differences between the wild-type and *tox* 176 sequences in the first 102 nucleotides, the plasmid was designated pTH1-176. The pSV2β-globin subcloning step introduced an additional *Bam*HI site, absent from pTH1 (13), as a *tox* 176 coding sequence-linked marker useful in confirming subsequent *tox* 176 expression plasmid constructs.

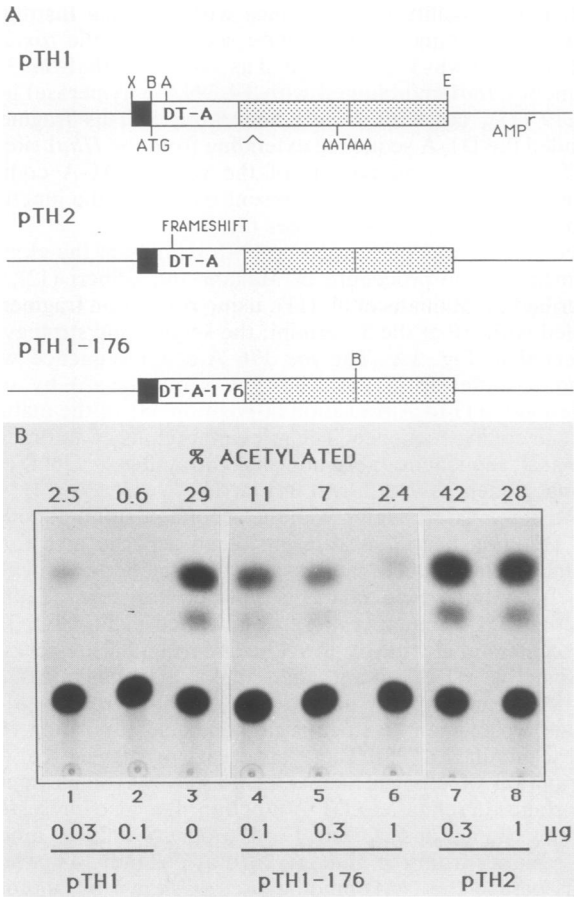


FIG. 2. (A) Structure of DT-A expression plasmids. In pTH1 the wild-type DT-A coding sequence, with downstream simian virus 40 splicing and polyadenylation signals (17), is attached to the truncated promoter region of the human metallothionein IIA gene (9). pTH2 (13) has a 2-base-pair insertion (frameshift) at the *Acc*I site in the DT-A coding sequence. pTH1-176 is identical with pTH1 except for the substitution of the *tox* 176 coding sequence and the presence of an additional *Bam*HI site, derived from pSV2 (17), which had been destroyed in the construction of pTH1 (13). The plasmid vehicle was pBR327. ——, pBR327 sequence; ■, metallothionein gene promoter; ⊠, simian virus 40 sequences; ATG, initiation codon: ⌒, small t splice (17); AATAAA, polyadenylation signal; AMP$^r$, β-lactamase gene. Restriction sites marked are as follows: X, *Xma*III; B, *Bam*HI; A, *Acc*I; E, *Eco*RI. (B) Assay of CAT activity in extracts of line 293 cells 48 h after transfection with pSV2cat (5 μg) together with the indicated quantities of DT-A expression plasmids. A control with 5 μg of pSV2cat alone is shown in lane 3. The autoradiogram of the thin-layer chromatogram (3) is shown. The lower spot is [$^{14}$C]chloramphenicol, and the two upper spots are monoacetylated chloramphenicol (isomeric forms of the reaction product) (3).

The effects of expression of wild-type and *tox* 176 DT-A in cultured human cells were compared by using a transient cotransfection assay as an indirect indication of toxicity (13). We previously showed, using this assay, that a DT-A expression plasmid, cotransfected with pSV2cat (3), substantially inhibited the expression of chloramphenicol acetyltransferase (CAT) activity. The inference that this resulted from inhibition of translation by DT-A was supported by observations that a frameshift mutation early in the DT-A coding sequence abolished the inhibition of CAT expression (13).

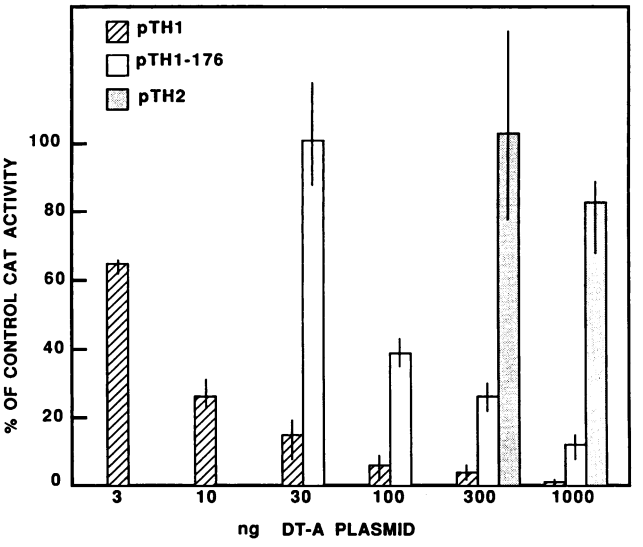


FIG. 3. Effect of cotransfection with DT-A expression plasmids on transient expression of CAT activity from pSV2cat by line 293 cells. Shown are the results of several experiments similar to that for which results are shown in Fig. 2B. The bars of the histogram each represent the average of two to five determinations whose range is indicated by the vertical lines.

The effect of replacing the wild-type DT-A coding sequence in the expression plasmid pTH1 (13) with that for *tox* 176 (plasmid pTH1-176) is illustrated in Fig. 2B, and the results of several such experiments are shown in Fig. 3. As reported previously (13), CAT expression from pSV2cat (5 μg) transfected into line 293 cells was strongly inhibited by minimal quantities of pTH1 (10 to 30 ng), whereas little or no inhibition occurred with as much as 1 μg of the DT-A frameshift plasmid pTH2 (Fig. 2B, lanes 1, 2, 7, and 8). pTH1-176 was also inhibitory to CAT expression (Fig. 2B, lanes 4 through 6), but approximately 15- to 30-fold more of this plasmid than of pTH1 was required for the same extent of inhibition (Fig. 3). These observations are consistent with reported estimates (19, 20) of the toxicity of *tox* 176 DT-A relative to that of the wild type. Although *tox* 176 DT-A has 8 to 10% of the in vitro ADP ribosylation activity of the wild type (19), it has been reported that 200 times more *tox* 176 DT-A than wild-type DT-A must be introduced into mouse L cells for lethality to occur (21). It is probably impossible to make precise comparisons among these estimates. For example, we have not established the relation of the amount of DT-A expressed to the input of expression plasmid, a determination which would be quite difficult for a product which inhibits its own synthesis. Also, although it is very likely that diminished toxicity is an inherent property of the *tox* 176 DT-A (19), our experiments did not exclude the additional possibility of decreased intracellular stability. In any case, it seems clear that the effective toxicity of the *tox* 176 product is diminished by 1 to 2 orders of magnitude relative to that of the wild-type DT-A. The cloned *tox* 176 DT-A coding sequence therefore promises to be a valuable tool in our attempts to obtain cell-type-specific suicide by toxin gene expression.

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