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Construction and Expression of Plasmids Containing Mutated Diphtheria Toxin A-Chain-Coding Sequences

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We previously demonstrated that cells can be killed through transfection of an expression plasmid that encodes the diphtheria toxin A-chain fragment (DT-A). This report describes the construction of expression plasmids containing three mutant DT-A-coding sequences substituting glutamic acid 148 with aspartic acid, serine, or glutamine which are known to have 100- to 300-fold-reduced ADP-ribosylation activity measured in vitro. The toxicity of these constructs was determined in cotransfection experiments using HeLa and 293 cells with a luciferase expression plasmid as the reporter. Dose responses were compared for the three new DT-A mutant plasmids and for the corresponding plasmids containing wild-type DT-A and the previously characterized *tox* 176 mutant. The dose required to produce 50% inhibition of control luciferase expression in 293 embryonic kidney cells for the five plasmids ranged from 0.01 μg for wild-type DT-A to 1.2 μg for the least toxic plasmid, which replaces glutamic acid 148 with glutamine. In conclusion, a wide range of DT-A toxicity can be achieved by using plasmid expression vectors that encode different DT-A mutations.

Diphtheria toxin (DT) consists of two functional units, the A and B chains, which produce toxicity to eukaryotic cells via B-chain-mediated binding to cellular receptors, internalization, and A-chain-mediated ADP-ribosylation of elongation factor 2 (4, 7, 15, 21). Wild-type DT is sufficiently toxic that a single molecule can cause cell death (22).

We have previously demonstrated, both in cell culture (11, 13) and in transgenic mice (2, 3, 14), that directed expression of the gene that encodes the DT A chain (DT-A) can result in selective destruction of a targeted cell population when the DT-A structural gene is linked with tissue-specific transcriptional regulatory elements. To address the potential problem of undesirable leaky expression which might produce toxicity in nontargeted tissues, mutants of DT-A with attenuated toxicity might be employed. Site-directed mutagenesis has been used to produce changes in the DT-A-coding sequence, resulting in mutants with reduced ADP-ribosylation activity as measured in vitro (18, 20). This report describes the construction of a series of expression plasmids containing these mutant coding sequences and assay of their activity upon transfection into two commonly used cell lines.

MATERIALS AND METHODS

DT-A plasmid constructions. Standard cloning methods were employed (10), and plasmids grown in *Escherichia coli* HB101 were isolated. Construction of pTH1, the prototype plasmid containing the wild-type DT-A-coding sequence linked to a truncated metallothionein promoter, has already been described (13). A DT mutant, *tox* 176, generated by Uchida et al. (19), was previously used in this laboratory to construct pTH1-176. Cotransfection experiments with 293 cells using a chloramphenicol acetyltransferase reporter plasmid showed diminished inhibition of chloramphenicol acetyltransferase expression compared with wild-type DT-A (11). One of us (R.J.C.) has recently generated three additional DT-A mutants by site-directed mutagenesis (18, 20), and these mutants were used as pBR322-derived bacterial expression plasmids (designated pTACF2E148D, pTAC F2E148S, and pTACF2E148Q) to construct the eukaryotic expression vectors described below. Reductions in ADP-ribosylation activity as measured in cell-free systems were 100-, 300-, and 250-fold for plasmids pTACF2E148D, pTAC F2E148S, and pTACF2E148Q, respectively (18, 20). These plasmids contain the full-length DT-A-coding sequence with mutations at the codon for amino acid 148 in DT-A. *Sau*3A1 sites are located 82 nucleotides 5′ of codon 1 for mature DT-A and at the last codon in the DT-A region. A 1,047-bp *Eco*RI-*Cla*I fragment was first isolated from each plasmid and digested with *Sau*3A1 to obtain the desired 659-bp fragment containing the mutated DT-A-coding sequence. This 659-bp fragment was subcloned into the *Bgl*II site of pSV2-327βG (13, 16), which was used as an intermediate because (unlike, e.g., pSV2neo) it retains a *Bgl*II site upstream of the simian virus 40 (SV40) small t splice and polyadenylation signal.

Construction of the pSV2-327βG derivative containing the pTACF2E148D, pTACF2E148S, or pTACF2E148Q fragment resulted in linkage of this fragment with the same downstream SV40 sequences as in the pTH1 expression plasmid. As the *Acc*I site in the DT-A coding sequence is 5′ to the mutations that we wished to transfer, the 1,602-bp fragment from the *Acc*I site through the SV40 downstream sequences to the *Apa*I site was substituted from the pSV2-327βG-based intermediate constructs into the pTH1 plasmid after the analogous fragment had been removed, giving rise to the plasmid series pTH1-E148D, pTH1-E148S, and pTH1-E148Q (see Fig. 1).

Cell culture. 293 cells are adenovirus-transformed human embryonic kidney cells (1, 6) obtained from J. Alwine and grown in Opti-MEM (GIBCO) with 3.8% fetal bovine serum. HeLa cells were maintained in Dulbecco's modified Eagle's

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medium with 10% newborn bovine serum. Cells were harvested for electroporation when cultures reached approximately 60% confluence.

Transient cotransfections. Cells were transfected by electroporation (12). Adherent cells were first released by trypsinization, washed once, and suspended in cold medium. A 100-μl cell suspension containing 5×10^6 cells was mixed with supercoiled plasmid DNA (in 5 μl of water) for electroporation in 0.4-cm presterilized disposable cuvettes (Bio-Rad). The DNA solutions contained 5 μg of the reporter plasmid, pSV2A/L-AΔ5′, a plasmid which produces large amounts of luciferase after transfection (5); the indicated amounts of the DT-A plasmid being tested; and a DT-A frameshift plasmid (pTH2) (13) as carrier DNA to render the total amount of DNA added constant. Capacitance settings on the Bio-Rad Gene Pulser were chosen so as to deliver a discharge with a time-constant readout of about 30 ms. The samples were then incubated at room temperature for 20 to 40 min before dilution into prewarmed medium and incubation at 37°C in an atmosphere containing 6% (vol/vol) CO_2. The cells were sampled and assayed for luciferase after a 24-h expression period (12).

Sequencing. The final expression plasmids were sequenced through the regions containing the mutated codon by using the method of Kraft et al. (8) for sequencing of double-stranded templates. Sequence data were generated by using the dideoxy method with Sequenase as the DNA polymerase (17). All sequences were as predicted by the construction strategy.

Statistics. The percentage of control luminescence observed by log dose linear relationships was tested for each of the plasmids. Linear regressions were used to estimate LI_{50} (50% luminescence inhibition) values for each plasmid and the associated 95% confidence intervals (9).

RESULTS

Figure 1 shows the important features of pTH1, the prototype DT-A expression plasmid, and demonstrates the position of the mutations in the E148 series plasmids, as well as the ADP-ribosylation activity of the DT-A mutant protein in cell-free assays (18, 20). The sequence of codon 148 in the final plasmid constructions was verified by independent sequencing.

Luciferase reporter activity was initially obtained with each plasmid, cotransfected at a dose of 300 ng plus 2,700 ng of plasmid pTH2, as well as control values obtained with 3,000 ng of plasmid pTH2 containing a frameshift mutation in the DT-A-coding sequence. Lysates from control cells produced 478,000 light U/100 μg of protein for HeLa cells and 162,000 light U/100 μg of protein for 293 cells. Inhibition of this expression by the various plasmids ranged from 23 to 72% in HeLa cells and from 22 to 74% in 293 cells.

Figure 2A and B are plots of the percentage of control luminescence against the log of the DNA dose in nanograms, in HeLa and 293 cells, respectively, while Table 1 provides an analysis of the LI_{50}s of different plasmids. As the figure illustrates, each expression plasmid demonstrated dose-dependent inhibition of luciferase expression over the range of 0.3 to 3,000 ng. The calculated slopes of these curves were significantly different ($P < 0.0001$ by the Procedure-General Linear Models from the Statistical Analysis System program [9]). The mutant plasmids showed attenuated levels of inhibition over this dose range compared with the wild-type activity of pTH1. The relative order of attenuated toxicity was nearly as predicted by reductions in ADP-ribosylation activity measured in cell-free systems, with the only discrepancy being that E148S was slightly more toxic than E148Q, while the opposite was true for the comparison of their ADP-ribosyltransferase activities (20).

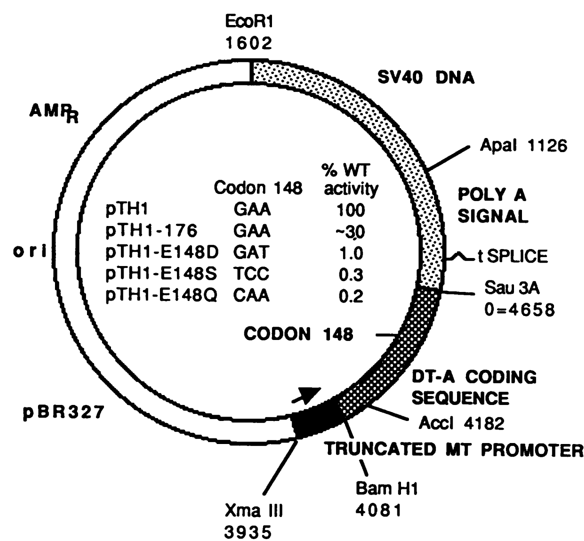

FIG. 1. Structure of plasmids expressing DT-A. Transfections were performed with covalently closed circular plasmids. The coding sequence for DT-A, with downstream SV40 splicing and polyadenylation signals (16), is joined to a 5′ truncated metallothionein (MT) promoter fragment (13) which also supplies the ATG initiation codon. Poly(A) signal (AATAAA), t splice (SV40 splice), and AMP_R (ampicillin resistance gene) locations are shown along with map positions and restriction enzyme sites. The codon sequence for position 148 is given. ADP-ribosylation activity is shown as previously reported (18, 20). WT, wild type; ori, origin.

DISCUSSION

The data presented in this report extend our observations on the use of DT mutants to obtain attenuation of toxicity of cellular expression vectors. The cotransfection assay provides a convenient means of testing transiently expressed mutant protein, since overall protein synthesis in cell cultures might be reduced to a lesser extent on the basis of efficiency of transfection.

Linear regression analysis also allowed us to estimate the plasmid LI_{50} (Table 1). These estimates gave a 39-fold difference and a 150-fold difference between the most toxic plasmid (pTH1) and the most attenuated mutant (pTH1-E148Q) in HeLa cells and 293 cells, respectively. The magnitude of reduction in toxicity for the codon 148 series of mutants was less than expected. The trivial explanation that mutation revertants or laboratory error could account for this phenomenon was eliminated by sequencing the mutation site of each of the final plasmid constructions (see Materials and Methods). ADP-ribosylation activity, as measured in cell-free assays, provides a measurement of enzyme activity, whereas cotransfection experiments introduce a number of additional variables. It is possible that the diphtheria toxin gene product controls its own synthesis in a feedback fashion, such that more of the attenuated DT-A protein is allowed to accumulate before it inhibits its own synthesis. This may allow the activity of the more attenuated toxin to increase relative to that of the wild-type toxin. It is possible

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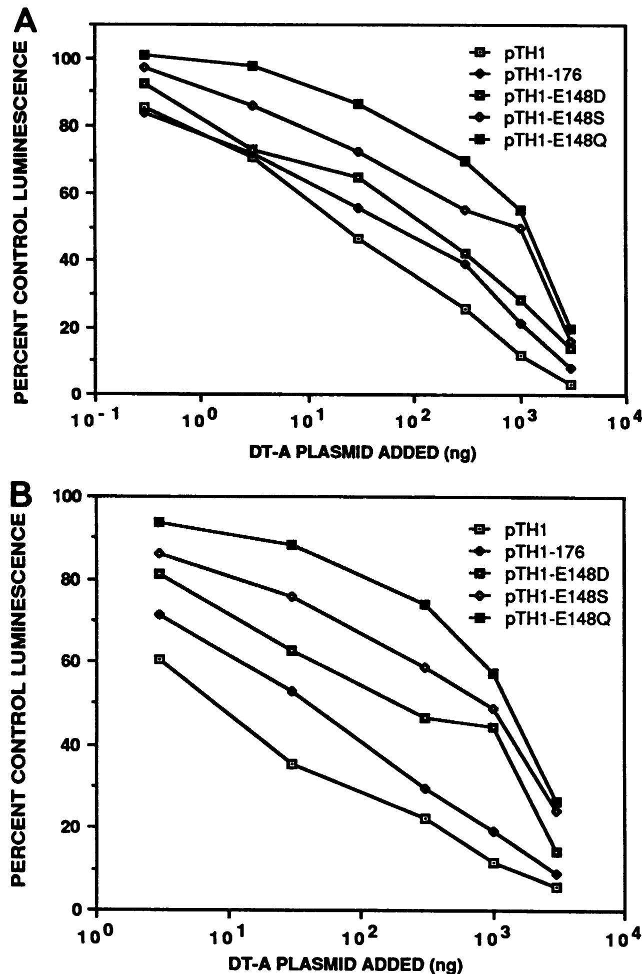

FIG. 2. Dose-dependent inhibition of luciferase reporter expression in HeLa (A) and 293 (B) cells. Each datum point represents the average percent inhibition of the control value for four separate transfections in HeLa cells and duplicate transfections in 293 cells. The results are expressed as percentages of the control luciferase activities observed in three parallel cotransfections with pSV2Aluc and the corresponding DT-A frameshift plasmid (pTH2; see Materials and Methods). Statistical analysis of these curves showed highly significant differences ($P < 0.0001$), with the confidence intervals shown in Table 1.

that E148Q protein is less stable than E148S in vivo, although we have no data concerning this possibility.

In previous work with *tox* 176 in transient cotransfection experiments with 293 cells, 30-fold more of the mutant plasmid was required to achieve similar levels of inhibition compared with wild-type DT-A (11). These experiments used calcium phosphate precipitation to introduce the plasmids into the cells. The results in this report demonstrate less difference in toxicity between *tox* 176 and wild-type DT-A in electroporated 293 cells. Therefore, the method used to introduce the DNA into the cells may also influence the apparent toxicity of DT-A mutants in a transient cotransfection assay.

Many potentially useful transcriptional control elements may not provide the exquisite control required to prevent toxicity in tissues not targeted for ablation. Attenuated toxins may allow the use of constructs containing regulatory

TABLE 1. Plasmid LI_{50}s for HeLa and 293 cells[a]

Plasmid	LI_{50} (μg) (95% confidence interval) for:	
	HeLa cells	293 cells
pTH1 (wild type)	0.019 (0.008–0.075)	0.008 (0.0006–0.014)
pTH1-176	0.035 (0.004–0.275)	0.034 (0.016–0.055)
pTH1-E148D	0.076 (0.005–0.193)	0.150 (0.030–3.000)
pTH1-E148S	0.230 (0.020–4.400)	0.440 (0.023–3.800)
pTH1-E148Q	0.740 (0.020–49.00)	1.200 (0.037–27.00)

[a] The LI_{50} was estimated by imposing linear models on each datum set. The linear regressions were used to calculate the LI_{50} of each plasmid. The 95% confidence intervals were approximated on the basis of the Statistical Analysis System Procedure-General Linear Models program (9) by using the prediction intervals provided by that routine.

elements which have some leakiness in nontargeted tissues. Results obtained with transgenic mice have demonstrated that toxin genes with reduced potency may produce milder phenotypic changes when substituted for wild-type DT-A. Breitman et al. recently combined the γ-2-crystallin regulatory sequences with *tox* 176, producing phenotypic patterns different from those produced by wild-type DT-A (3). Also, attempts to produce transgenic animals by using the promoter region for myelin basic protein were successful with the *tox* 176 but not with the wild-type DT-A-coding sequence (results cited in reference 3). The results reported here suggest that DT-A mutants with even further attenuation of toxicity might be useful reagents in similar tissue-specific expression experiments.

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