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# Regulated expression of the diphtheria toxin A chain by a tumor-specific chimeric transcription factor results in selective toxicity for alveolar rhabdomyosarcoma cells

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**ABSTRACT** **Alveolar rhabdomyosarcoma (ARMS) cells often harbor one of two unique chromosomal translocations, either t(2;13)(q35;q14) or t(1;13)(p36;q14). The chimeric proteins expressed from these rearrangements, PAX3-FKHR and PAX7-FKHR, respectively, are potent transcriptional activators. In an effort to exploit these unique cancer-specific molecules to achieve ARMS-specific expression of therapeutic genes, we have studied the expression of a minimal promoter linked to six copies of a PAX3 DNA binding site, prs-9. In transient transfections, expression of the prs-9-regulated reporter genes was ≈250-fold higher than expression of genes lacking the prs-9 sequences in cell lines derived from ARMS, but remained at or below baseline levels in other cells. High expression of these prs-9-regulated genes was also observed in a cancer cell line that lacks t(2;13) but was stably transfected with a plasmid expressing PAX3-FKHR. Transfection of a plasmid containing the diphtheria toxin A chain gene regulated by prs-9 sequences (pA3–6PED) was selectively cytotoxic for PAX3-FKHR-expressing cells. This was shown by inhibition of gene expression from cotransfected plasmids and by direct cytotoxicity after transfected cells were isolated by cell sorting. Gene transfer of pA3–6PED may thus be useful as a cancer-specific treatment strategy for t(2;13)- or t(1;13)-positive ARMS. Furthermore, gene transfer of fusion protein-regulated toxin genes might also be applied to the treatment of other cancers that harbor cancer-specific chromosomal translocations involving transcription factors.**

Rhabdomyosarcoma is the most common soft tissue sarcoma of childhood. While implementation of surgery, radiation, and chemotherapy has improved the overall long-term survival rate, patients often suffer numerous acute and chronic sequelae (1). In addition, those with metastatic disease or an unfavorable histological subtype continue to have a poor prognosis (2). Identification of one of these high risk types, alveolar rhabdomyosarcoma (ARMS), has been facilitated by cytogenetic and molecular detection of the characteristic chromosomal translocations t(2;13)(q35;q14) or t(1;13)(p36;q14).

The t(2;13)(q35;q14) or t(1;13)(p36;q14) fusion genes encode hybrid proteins derived from the DNA-binding domains of PAX3 or PAX7, respectively, and the transactivation domain of an unrelated transcription factor, FKHR (3–6) (Fig. 1). PAX3-FKHR is ≈100-fold more potent than wild-type PAX3 in transactivating genes linked to PAX3 response elements, such as the e5 element found in the *Drosophila even-skipped* promoter (7, 8). Both the paired box and homeobox elements in e5 that interact with the paired box domain and homeodomain of PAX3, respectively, are required for efficient PAX3 binding (Fig. 1). PAX3 binds the paired domain recognition sequence (prs)-9, a synthetic derivative of e5, more efficiently than it binds e5 (9).

PAX3 and PAX7 are normally expressed during murine embryogenesis in developing neural and muscle tissues (10). Although some investigators have not detected expression of these genes in adult tissues (10), others have detected low levels in subregions of the adult mouse brain (11). The pattern of expression of PAX3 and PAX7 in adult humans, if any, has not yet been determined. The inappropriate expression of PAX3 in the context of the PAX3-FKHR fusion protein is thought to contribute to the pathogenesis of ARMS by activating other cellular genes normally dormant in the differentiated muscle cell. Among the possible consequences of such dysregulated gene expression, PAX3-FKHR expression may contribute to cellular immortality by inhibiting apoptosis, or programmed cell death (12).

Protein domain swapping between unrelated transcription factors similar to that seen in PAX3-FKHR and PAX7-FKHR is also observed in other fusion proteins found in many human neoplasms and leukemias (13). The DNA sequence at the translocation breakpoint and the expressed chimeric mRNA and protein are thus cancer-specific molecules. Efforts to exploit such molecules for therapy have been limited to strategies using antisense oligonucleotides (14), ribozymes (15, 16), and peptides (17) to recognize the unique junctional DNA or mRNA sequence and thereby inhibit fusion gene expression. An alternative approach to exploiting these genetic rearrangements would be to capitalize on any unique function(s) of the fusion proteins, such as the potent transcriptional activation properties of PAX3-FKHR. PAX3 responsive elements (such as the strong-binding prs-9 site) that mediate transactivation by PAX3-FKHR might thus be useful in selectively expressing exogenous therapeutic genes in ARMS cells.

The bacterial protein diphtheria toxin A chain (DT-A) catalytically ADP ribosylates the diphthamide group of cellular elongation factor 2, inhibiting protein translation and activating apoptosis, or programmed cell death (18, 19). Although diphtheria toxin is highly toxic to eukaryotic cells (20), transgenic mice containing the DT-A gene under the control of different tissue-specific promoters suggest that expression of

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Abbreviations: ARMS, alveolar rhabdomyosarcoma; prs, paired domain recognition sequence; β-gal, β-galactosidase; RT, reverse transcription; DT-A, diphtheria toxin A chain; DT, diphtheria holotoxin; CAT, chloramphenicol acetyltransferase.

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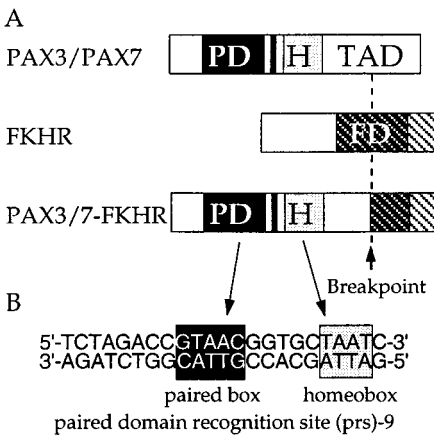


FIG. 1. (*A*) Structure of PAX3/PAX7, FKHR, and PAX3/7-FKHR. The paired domain (PD), homeodomain (H), and octapeptide (solid bar between PD and H) of PAX3/PAX7 as well as the transcriptional activation domain (TAD) of FKHR are conserved in PAX3/7-FKHR, but the fork-head DNA-binding domain of FKHR (FD) and the TAD of PAX3/7 are disrupted. Expression of PAX3-FKHR or PAX7-FKHR is limited to ARMSs containing t(2;13)(q35;q14) or t(1;13)(p36;q14), respectively. (*B*) PAX3 DNA binding site. The core DNA motifs bound by PAX3/7 and PAX3/7-FKHR are present in the paired domain recognition site (prs)-9 that is derived from the e5 sequence in the *Drosophila even-skipped* promoter.

DT-A can be controlled without unintended toxicity to other cells (21–24). Indeed, selective expression of transfected DT-A genes has been proposed as a possible strategy to target cancer cell killing (25). Whether such stringent tissue-specific regulation of expression can be achieved after transient transfection so that only targeted cells are killed, however, has not yet been determined.

Using the PAX3-FKHR molecule expressed in t(2;13)-positive ARMS as a model of cancer-specific chimeric fusion proteins, we show here that controlled selective expression of exogenous genes in cells expressing PAX3-FKHR can be achieved using a minimal promoter sequence (E1bTATA) linked to six tandem copies of prs-9. The 6xprs-9-E1bTATA promoter was capable of tightly regulating expression of the diphtheria toxin A chain gene after transient transfection, resulting in specific cytotoxicity for ARMS cells.

## MATERIALS AND METHODS

**Cells and Cell Cultures.** Human rhabdomyosarcoma cell line RD and cervical carcinoma cell line HeLa were obtained from the American Type Culture Collection. Rh18 was kindly provided by Peter Houghton (St. Jude's Children's Research Hospital, Memphis, TN). FMD24#3 and PCD3M8#13 were derived from RD cells stably transfected with pCDNA3 (control) or pCMV-*pax3-fkhr*, respectively. Primary human fibroblasts were grown from a necrotic tumor biopsy sample. Media were obtained from Fisher Scientific. Cells were cultured in RPMI 1640 medium (Rh18, fibroblasts), DMEM (RD), DMEM supplemented with 0.4 mg/ml geneticin (PCD3M8#13, FMD3#24) or MEM (HeLa) each containing 10% fetal bovine serum (HyClone) and 100 $\mu$g penicillin and 100 units streptomycin per ml. All cells were subcultured prior to confluence. DT holotoxin was obtained from Sigma and added directly to the medium.

**Plasmids.** pSV2-$\beta$-galactosidase ($\beta$-gal) was purchased from Promega and pEGFP from CLONTECH. pTk-*cat*-261 (26) was kindly provided by P. Gruss (Gottingen, Germany) and pCMV-*lacZ* by Hans Herweijer (Waisman Center, Madison, WI). p6xPRS9-E1bTATA-*cat* (8) and pCMV-*pax3-fkhr* (7) have been described. The poly(A) trimer derived from SV40 was cloned as a *Hin*dIII fragment from pTHA7 (27) upstream of the prs-9 sites in p6xPRS9-E1bTATA-*cat* (p6PEC) to yield pA3–6xPRS9-E1bTATA-*cat* (pA3–6PEC). The *cat* gene in p6PEC was substituted with the DT-A gene by cloning a 2.2 kb *Eco*RI fragment from pTHA7$\Delta$H (pTHA7 with the downstream *Hin*dIII site filled in creating a *Nhe*I site) into p6PEC using an *Eco*RI linker at a downstream *Hpa*I site, yielding p6xPRS9-E1bTATA-dta (p6PED), and the *Hin*dIII fragment encompassing A3 was cloned into the *Hin*dIII site to yield pA3–6PED. The DT-A coding sequence in this construct comprises amino acids 3–193 of wild-type DT-A with a 24 amino acid C-terminal extension that does not interfere with its activity, as described (21).

**Lipofection.** Cells were plated at 2–4 $\times$ $10^5$ cells per well in 6-well dishes one day prior to transfection. 3 $\mu$l lipofectamine (GIBCO/BRL) and 1 $\mu$g total plasmid DNA was used in serum-free medium according to the manufacturer's recommendations. In primary fibroblasts (Fig. 2) addition of a replication-defective adenovirus expressing the luciferase reporter gene, Ad*lux* (28), kindly provided by Hans Herweijer, was added at a multiplicity of infection of 20 to achieve higher gene transfer efficiency (29). After 2–6 hr, an equal volume of medium containing 20% fetal bovine serum was added. At 24 hr, the medium was replaced by fresh complete medium. Cells were assayed at 48 hr postinfection except for those in Figs. 4 *B* and *C*, which were harvested at 12–16 hr, a time of maximal expression of $\beta$-gal when cotransfected with plasmids expressing DT-A. In Fig. 4*B*, cells were cotransfected with 0.9 $\mu$g pCMV-*lacZ* plus 0.1 $\mu$g of either pA3–6PEC or pA3–6PED, and in Fig. 4*C* they were cotransfected with 0.5 $\mu$g pEGFP plus 0.5 $\mu$g of either pA3–6PEC or pA3–6PED.

**Cell Sorting.** For each data point in Fig. 4 *B* and *C*, six 9.5 $cm^2$ wells of transfected cells were harvested in trypsin/EDTA, pooled, pelleted at 500 $\times$ *g*, and resuspended in 100 $\mu$l complete medium at 37°C. In Fig. 4*B*, 100 $\mu$l of fluorescein di-$\beta$-D-galactopyranoside (Molecular Probes) at 2 mM in 98% $H_2O$, 1% dimethyl sulfoxide, 1% ethanol, and 200 $\mu$M verapamil (to prevent fluorescein efflux; ref. 30) was prewarmed to 37°C and added to the cells. After 1 min at 37°C, the cells were diluted and chilled rapidly with 1 ml ice-cold complete medium and incubated at 4°C for 30 min. Cells were rinsed once with ice-cold complete media containing 100 $\mu$M verapamil and resuspended in the same media/verapamil containing 50 $\mu$M propidium iodide (to exclude dead cells in the sort). For Fig. 4*C*, cells were rinsed in complete media twice and resuspended in complete media containing 50 $\mu$M propidium iodide. For both Figs. 4 *B* and *C*, the cells were passed through a 40 $\mu$m Nytex mesh (Tetko, Elmsford, NY), loaded onto a FACSTAR cell sorting flow cytometer, and analyzed using CELLQUEST software (Becton Dickinson) at a flow rate of 2,000 cells/sec. Analysis and sorting regions were set to quantify and isolate living cells with fluorescein signals higher than the background cellular autofluorescence measured in untransfected cells. Equal numbers (5,000–11,000) of living cells (determined by trypan blue exclusion after sorting) were plated in three to nine wells of a 96-well tissue culture plate with complete medium and allowed to grow from 1–4 days before assessing for cellular viability by the CellTiter 96 assay (Promega) according to the manufacturer's recommendations.

**Reporter Gene Expression.** Chloramphenicol acetyltransferase (CAT) expression was detected using a modified organic extraction (31). Briefly, cells were rinsed in PBS three times and harvested in 1.2 ml TEN buffer (40 mM Tris·HCl, pH 7.5/1 mM EDTA, pH 8.0/150 mM NaCl). Extracts were prepared in 100 $\mu$l 0.25 M Tris (pH 8.0) by three cycles of rapid freeze/thaw in liquid nitrogen and stored at $-80$°C. Debris was pelleted at 13,000 $\times$ *g* for 5 min. For overnight incubations, extracts were heated at 60°C for 10 min. CAT reactions were assembled as follows: 5–60 $\mu$l cell extract, 0.44 mg/ml butyryl-CoA (Sigma), 150 mM Tris, pH 8.0, and 0.075 $\mu$Ci D-

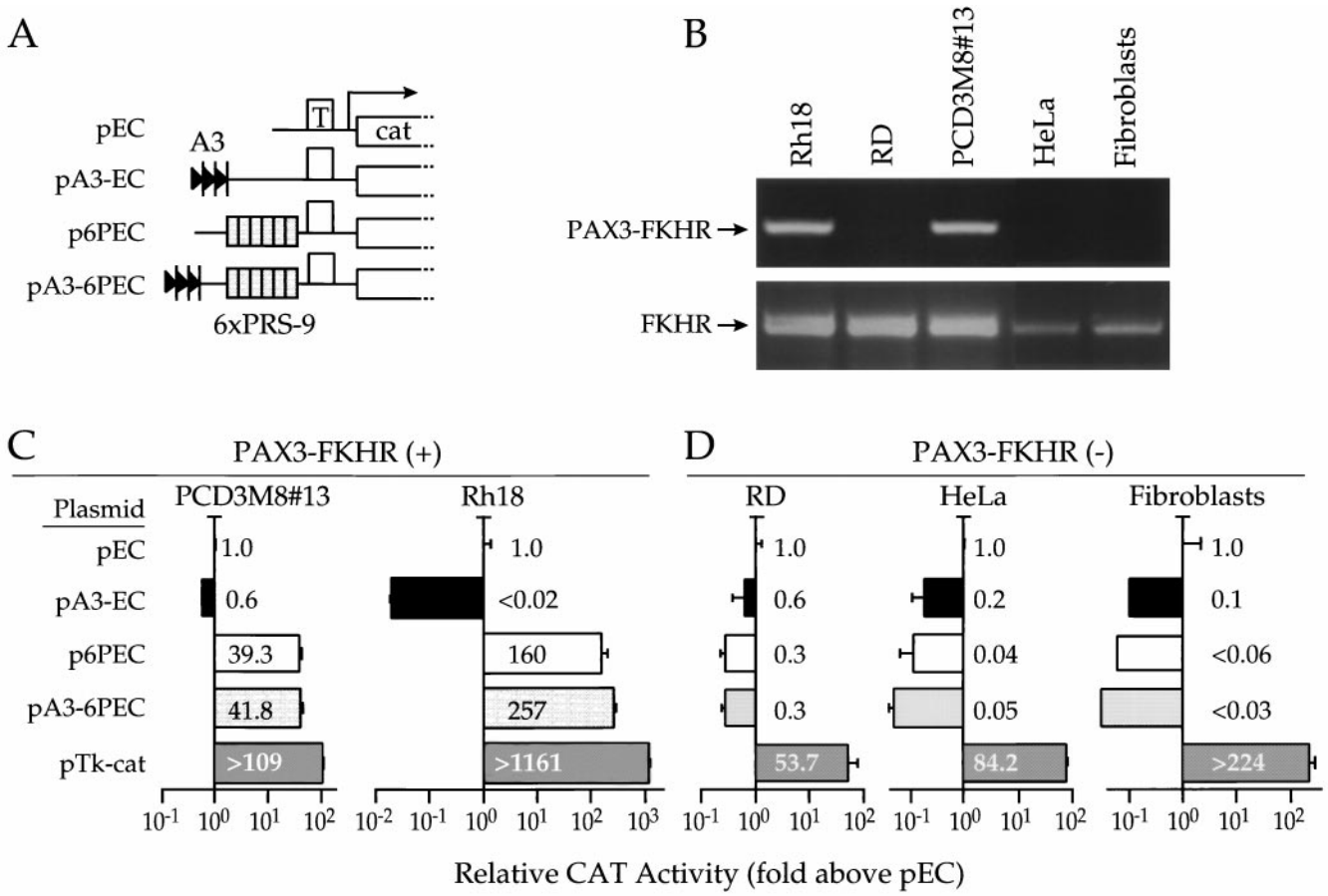


FIG. 2. Effect of prs-9 and upstream polyadenylation sites on reporter gene expression in different cells. (*A*) Schema of four separate reporter plasmids used in these studies. A3, three tandem copies of the simian virus 40 early region polyadenylation signal; prs-9, paired box recognition sequence-9; T, 12-mer oligonucleotide encompassing the TATA box from the adenovirus E1b promoter. The arrow indicates the direction of transcription. (*B*) RT-PCR of PAX3-FKHR. Internal primers for the ubiquitous FKHR gene were used as controls for each RNA sample. (*C, D*) Relative expression of CAT after transient transfection in PAX3-FKHR(−) and PAX3-FKHR(+) cell lines. In addition to the plasmids shown in *A*, pTk-*cat*-261 was used as a positive control for *cat* gene transfection. CAT activity is expressed relative to that measured for pEC in each cell line and determinations were performed in triplicate. Numbers are averages. Bars = mean ± SD. Values shown as "greater than" were beyond the linear range of the assay, and values shown as "less than" were undetectable (not above the background detected in cells transfected with pUC19 or herring sperm DNA, generally ≈25–50 dpm).

threodichloroacetyl-1-$^{14}$C]chloramphenicol (Amersham 1 Ci = 37 GBq) in a total volume of 180 μl. After 2–24 hr at 37°C, reactions were extracted with mixed xylenes (Sigma) and back-extracted twice with 0.25 M Tris, pH 8.0. The remaining xylene phase was counted in 4 ml ScintiSafe Plus 50% scintillation fluid using a Beckman LS6000 scintillation counter (Beckman Instruments). The disintegrations per minute measured in the control transfection with pUC19 (lacking a *cat* gene) or Herring sperm DNA in each experiment was subtracted from all values. Standard CAT curves were included in each experiment to ensure that measured values were within the linear range of the assay. For β-gal detection, the Galacto-Light Plus chemiluminescent reporter assay (Tropix, Bedford, MA) was used in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego) according to the manufacturer's recommendations.

**Reverse Transcription (RT)–PCR.** RNA samples were prepared from cell extracts using the QIAshredder and RNeasy protocol according to manufacturers recommendations (Qiagen, Chatsworth, CA). RT-PCR was performed using a modified version of the Access RT-PCR system (Promega) with 1 mM $MgSO_4$. Oligonucleotides 5′ PAX3/7–1 and 3′ FKHR RT primer spanning PAX3-FKHR have been described (32). Internal FKHR primers were "F1" 5′-GCTACTCGTTTGC-GCCACCAAAC-3′ and "FR2" 5′-CCGTGTGGGGCAGG-GGACG-3′ yielding a predicted length of 394 bp. 1 μg total cellular RNA was first heated to 75°C for 3 min and placed on ice. Reverse transcription was accomplished at 48°C for 45 min followed by 94°C for 2 min. Amplification reactions were carried out for 35 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min followed by a final extension at 72°C for 7 min and visualized on a 2% agarose gel with ethidium bromide.

## RESULTS

**PRS-9-Dependent Gene Expression.** Tandem repeats of prs-9 sites linked to a TATA box derived from the adenovirus E1b promoter in the plasmid p6xPRS9-E1bTATA-*cat* (p6PEC; Fig. 2*A*) have been shown previously to mediate transcriptional activation by pCMV-*pax3-fkhr* in transient co-transfections (8). To determine if the prs-9-mediated activation can be observed in cells that endogenously express PAX3-FKHR, p6PEC was tested in several cell lines that differ with respect to PAX3-FKHR expression. The cell line RD was derived from an embryonal rhabdomyosarcoma that lacks t(2;13) and does not express PAX3-FKHR. PCD3M8#13 is a stable pCMV-*pax3-fkhr* transfectant of RD, and Rh18 is a t(2;13)-positive cell line derived from an ARMS. Expression of PAX3-FKHR transcripts was confirmed to be present in Rh18 and PCD3M8#13 cells and absent in RD, HeLa, and primary fibroblasts by RT-PCR (Fig. 2*B*). As shown in Fig. 2*C*, the six copies of prs-9 resulted in a stimulation of transcription above

the minimal promoter (pEC) 39.3- and 160-fold, respectively, in the two PAX3-FKHR(+) cell lines.

To eliminate any read-through translation of mRNAs arising from cryptic plasmid promoters, a trimer tandem repeat of the simian virus 40 early polyadenylation signal (A3) was added upstream of the E1bTATA box and prs-9 sites in pEC and p6PEC, generating pA3-EC and pA3–6PEC, respectively (Fig. 2*A*). In all cell lines tested, the A3 sequence reduced the baseline expression of pEC. In fact, expression of pA3-EC in Rh18 cells was not detectable above the background pUC19 control. The A3 sequence did not significantly affect the activity of the prs-9-linked promoter that was high in Rh18 and PCD3M8#13 cells and was repressed to near background levels in RD and HeLa cells (Fig. 2 *C* and *D*). When compared with pA3-EC, the addition of prs-9 sites in pA3–6PEC resulted in a >12,000-fold augmented expression of CAT in Rh18 cells and a 70-fold increase in PCD3M8#13 cells. A consistent transfection efficiency for each CAT plasmid was demonstrated by measuring β-galactosidase activity in lysates from cells cotransfected with pCMV-*lacZ* (not shown). pCMV-*lacZ* was not included in the experiments shown because of marked squelching of CAT expression that resulted from the cotransfections.

**Sensitivity of PAX3-FKHR(+) Cells to Diphtheria Holotoxin (DT).** Because inhibition of PAX3-FKHR expression by antisense oligonucleotides can trigger ARMS cells to undergo apoptosis (12), apoptosis-resistance may be one of the possible mechanisms underlying unregulated ARMS cell growth. We therefore sought to determine whether ARMS cells are sensitive to the cytotoxic effects of exogenously added DT. Cells were first exposed to 1,000 ng/ml DT and followed daily for survival by the CellTiter 96 assay. As shown in Fig. 3*A*, all cell lines tested were sensitive to the toxic effects of DT. The effect of DT exposure was relatively slow, requiring 4–5 days for the more sensitive lines to be completely killed. Maximal toxicity in Rh18 cells was not seen until 6 days. To determine the relative sensitivity of these cells to DT, dose-response curves spanning five logs of DT concentration were conducted and the relative cell survival measured after 6 days. Fig. 3*B* demonstrates that only at the lower concentrations of DT did Rh18 appear somewhat less sensitive to the effects of DT. Furthermore, the cell line PCD3M8#13, which is the stable transfectant of RD with pCMV-*pax3-fkhr*, was equally sensitive to DT as the control transfectant of RD, FMD24#3, which does not express PAX3-FKHR. These results suggest that expression of PAX3-FKHR does not significantly alter the sensitivity of cells to DT-mediated cytotoxicity.

**Cell-Specific Cytotoxicity of Regulated DT-A Expression.** HeLa and RD cells were chosen as the PAX3-FKHR(−) cell lines for cytotoxicity studies because of relatively high transfection efficiencies. In addition, while HeLa does not express any detectable PAX3 by RT-PCR (data not shown), RD is a tumor cell line that expresses wild-type PAX3 (12), and is thus a model of cells that express PAX3 but not PAX3-FKHR. Cell-specific toxicity of the gene encoding DT-A regulated by the 6xPRS-9 sequence (pA3–6PED) was tested by measuring both inhibition of the expression of a cotransfected plasmid and direct cytotoxicity. For translation inhibition, HeLa and Rh18 cells were transiently cotransfected with 0.5 μg pSV2-*βgal* and various amounts of pA3–6PED (pA3–6PEC was included to avoid squelching of transcription factors by keeping the total amount of promoter sequences constant). Two days after transfection, ≈60% inhibition of β-gal expression was detected in Rh18 cells even at a 1:10 ratio of pA3–6PED:pSV2-*βgal* (0.05 μg pA3–6PED, Fig. 4*A*). Inhibition of β-gal was more dramatic with higher amounts of pA3–6PED in Rh18 cells, without significantly affecting β-gal expression in HeLa cells. Similar results were obtained with the inhibition of CAT expression from pTk-*cat* by pA3–6PED (not shown).

To demonstrate that the inhibition of translation in Rh18 corresponds to cell death after toxin gene transfer, transfected cells were sorted from untransfected cells and followed in culture for growth. 12–16 hours after cotransfection with 0.9 μg pCMV-*lacZ* (used for cell sorting) and 0.1 μg pA3–6PED or pA3–6PEC (control), Rh18 and HeLa cells were incubated with fluorescein-di-β-D-galactopyranoside, subjected to fluorescence-activated cell sorting (to select the β-gal positive transfected cells), and replated. Staining of cells with 5-bromo-4-chloro-3-indolyl-β-D-galactoside 1 day after sorting showed that only ≈90% of both DT-A and *cat* transfected cells in the positive sort expressed β-gal (not shown). This may have resulted in part from passive intercellular transfer of fluorescein prior to the sort resulting in ≈10% false-positive cells or perhaps from autofluorescence. Cultures were followed longitudinally for cell growth. The relative growth of cells transfected with the DT-A plasmid pA3–6PED is shown compared with the growth of those transfected with the control plasmid pA3–6PEC to control for any nonspecific toxic effect of transfection and cell sorting (Fig. 4*B*). As shown, transient transfection of pA3–6PED resulted in a dramatic inhibition of growth in Rh18 cells but not in HeLa cells. These results were consistent with those obtained on two separate repetitions of this experiment. Similar results were obtained in a separate experiment in RD and Rh18 cells when cell sorting was based on expression of green fluorescent protein instead of β-gal (Fig. 4*C*). Again, there were 5–10% negative cells in the positive transfectants when examined under fluorescent microscopy immediately after sorting, perhaps due to autofluorescence or photobleaching. Nevertheless, we measured significant growth inhibition in cells transfected with pA3–6PED relative to those transfected with pA3–6PEC for PAX3-FKHR(+) Rh18 cells but not for PAX3-FKHR(−), PAX3(+) RD cells.

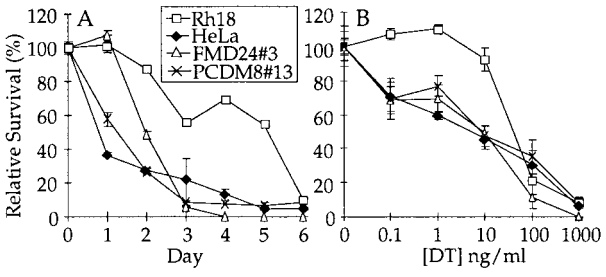


FIG. 3. Relative survival after exposure to DT of two cell lines that lack PAX3-FKHR expression (HeLa, FMD24#3) and two cell lines that express PAX3-FKHR (Rh18, PCD3M8#13). (*A*) Time course of DT toxicity. Cells were exposed in triplicate to 1,000 ng/ml DT and followed daily for survival. Bars = Mean ± SD relative to cultures at day 0 before DT was added. (*B*) DT dose response curve. Cells were exposed in triplicate to various concentrations of DT. After 6 days, survival was determined and is shown relative to the control culture without DT.

## DISCUSSION

The high-risk childhood cancer ARMS expresses the transcriptional fusion proteins PAX3-FKHR or PAX7-FKHR. We have demonstrated that a plasmid construct (pA3–6PED) containing the DT-A gene under the control of a minimal TFIID binding site linked to upstream PAX3 binding sites and a trimer of polyadenylation signals is selectively cytotoxic for cells expressing PAX3-FKHR. After transient transfection, pA3–6PED was sufficiently active in cells expressing PAX3-FKHR to cause cell death without adversely affecting cells that do not express PAX3-FKHR (HeLa and RD). Such controlled gene expression is therefore an effective way of achieving selective expression of therapeutic genes in ARMS cells. Although previous attempts to accomplish cancer-specific

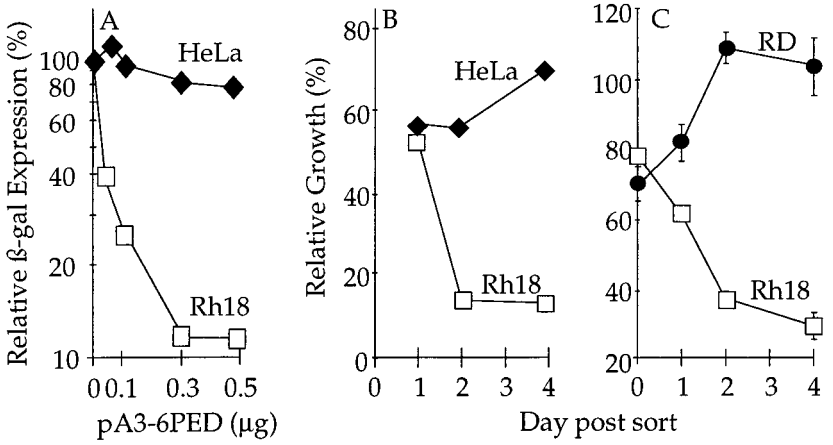


FIG. 4. Cell-selective toxicity of pA3–6PED. (*A*) ARMS cells (Rh18) or cervical carcinoma cells (HeLa) were transiently cotransfected with 0.5 μg pSV2-βgal and increasing concentrations of pA3–6PED. β-Gal activity was determined after 48 hr and is expressed relative to the β-gal measured in cells cotransfected with pA3–6PEC. Numbers are averages of duplicate points. (*B*) Positive transfectants of Rh18 and HeLa cells as determined by fluorsecence-activated cell sorter-fluorescein di-β-D-galactopyranoside analysis of β-gal expression were sorted from negative cells, replated in 96 well dishes, and followed for cell proliferation. (*C*) Positive transfectants of Rh18 cells (expressing PAX3-FKHR) and RD cells (expressing PAX3 but not PAX3-FKHR) as determined by expression of green fluorescent protein were sorted from negative cells and followed for cell growth. In both *B* and *C*, growth of cultures transfected with the DT-A plasmid pA3–6PED is shown relative to the growth of cultures transfected with the control plasmid pA3–6PEC. In *C*, averages of triplicate points are shown with SD where visible outside of the data symbol. Rh18 cells, □; HeLa cells, ◆; RD cells, ●.

gene expression have focused on utilizing normal gene promoters that are up-regulated in different cancers (33), this is the first report of exploiting a cancer-specific genetic rearrangement to activate a therapeutic gene.

While PAX3-FKHR is a strong transactivator, the normal PAX3 and PAX7 proteins should also be able to transactivate DT-A expression from pA3–6PED, although ≈100-fold less efficiently. In addition to PAX3 and PAX7, the prs-9 element also mediates transactivation by PAX1 (26). Because expression of DT-A from pA3–6PED is driven by tandem repeats of prs-9, there may be some tumor cells where PAX1, PAX3, or PAX7 are up-regulated (by mechanisms other than gene fusions) to levels sufficient to express toxic levels of DT-A from transfection of pA3–6PED. While PAX3 is expressed in the embryonal rhabdomyosarcoma cell line RD (12), we did not detect any stimulation of expression of pA3–6PEC in these cells (Fig. 2*D*) and transfection of pA3–6PED in them was not cytotoxic (Fig. 4*C*). PAX3 expression may be relatively low in RD, however, and higher in other tumor cells. In fact, PAX1 and/or PAX3 expression has been shown by others to be markedly elevated in 10 of 30 medulloblastomas (34). Whether pA3–6PED is also a "selectively toxic plasmid" for other such cancer cells that aberrantly express these PAX proteins remains to be determined.

In contrast to antisense strategies that require that down-regulation of the targeted gene inhibits cell growth or triggers apoptosis, the strategy of regulated DT-A gene expression only requires that fusion protein expression be preserved. Indeed, cancer cells are genetically heterogeneous and often acquire many different mutations. In particular, ARMS cells are prone to genomic amplifications (35). While it is therefore possible (through the acquisition of other mutations) that ARMS cells might no longer require expression of the fusion protein to maintain a malignant phenotype, PAX3-FKHR has been detected in ARMS metastases (36) and relapsed specimens (not shown), suggesting that its expression may be necessary to remain cancerous.

Currently, many protocols of gene therapy for cancer utilize the gene encoding the drug-converting enzyme derived from herpes simplex virus, thymidine kinase (37). After administration of acyclovir or ganciclovir, phosphorylated products of these drugs are made in cells expressing thymidine kinase and inhibit cellular DNA replication. The active drug is capable of traversing intercellular gap junctions (if present) and affecting adjacent cells that do not express the foreign gene (bystander effect) (38). Use of the gene encoding diphtheria toxin, which does not exhibit a bystander effect, might be desirable in certain settings such as *in vitro* autologous bone marrow purging of contaminating tumor cells (39) and widely metastatic disease where the bystander effect might be harmful to surrounding normal cells. Because of its potency, the DT-A gene might also be advantageous in settings where only low levels of gene expression are possible. Finally, unintended toxicity from basal expression in nontargeted cells might be avoided by using less potent, attenuated versions of DT-A (40).

These studies demonstrate that chimeric transcription factors found in ARMS can be exploited to achieve highly cell-specific expression of exogenous "therapeutic" genes *in vitro*. A similar strategy should be possible with other cancers or leukemias that express distinct fusion proteins that function as tumor-specific transcription factors (13). Even if expression in these cancers is selective but not completely specific, the combination of such "transcriptional targeting" with tissue-specific delivery by modified viral vectors (41) or peptide-presenting phage (42) may result in very highly specific exogenous gene expression.

Toxin gene therapy for cancer does not share the same limitations as gene therapy for genetic diseases. Whereas most gene therapy strategies aimed at correcting a genetic defect require prolonged, high levels of foreign gene expression, toxin gene therapy for cancer allows for relatively low, transient levels of exogenous gene expression to achieve the desired cytotoxicity. Because of its cell specificity, targeted gene expression as a form of cancer therapy may exhibit fewer short and long-term side effects than conventional therapies. In addition, the systemic and local intratumoral immunosuppression of conventional chemotherapy and radiotherapy might be avoided, which in certain clinical settings may permit a more effective anti-tumor host immune response.

The primary limiting factor for toxin gene therapy of cancer remains the current inability to transfect all clonogenic tumor cells at primary or metastatic sites *in vivo*. Because the fidelity of heterologous tissue-specific gene expression can be retained in the context of viral genomes (43), delivery of therapeutic genes under the control of the A3–6xPRS-9 E1bTATA promoter via replication-defective adenoviruses or retroviruses

might be an effective approach for high efficiency *in vivo* gene transfer. Therefore, as gene transfer technology becomes more refined, the functional properties of chimeric transcription factors such as PAX3-FKHR might be harnessed to specifically activate exogenous genes for use as cancer-specific therapeutic strategies.

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