doi:10.1016/S1525-0016(03)00056-X

Inhibition of Tumor Growth by DT-A Expressed Under the Control of IGF2 P3 and P4 Promoter Sequences

B. Ayesh,[1,*] I. Matouk,[1] P. Ohana,[1] M. A. Sughayer,[2] T. Birman,[3] S. Ayesh,[1] T. Schneider,[1] N. de Groot,[1] and A. Hochberg[1]

[1]*Department of Biological Chemistry, Institute of Life Sciences, Hebrew University, Jerusalem 91904, Israel*
[2]*Department of Pathology, Makassed Hospital and Alquds University School of Medicine, Jerusalem, Israel*
[3]*Department of Pathology, Hadassah University Hospital, Jerusalem, Israel*

**To whom correspondence and reprint requests should be addressed. Fax: 972-2-5610250. E-mail: Basim@vms.huji.ac.il.*

The human IGF2 P3 and P4 promoters are highly active in a variety of human cancers. We here present an approach for patient oriented therapy of TCC bladder carcinoma by driving the diphtheria toxin A-chain (DT-A) expression under the control of the IGF2 P3 and P4 promoter regulatory sequences. High levels of IGF2 mRNA expression from P3, P4 or both promoters were detected in 18 TCC samples (n = 29) by ISH or RT-PCR. Normal bladder samples (n = 4) showed no expression from either promoter. The activity and specificity of the IGF2 P3 and P4 regulatory sequences were established in human carcinoma cell lines by means of luciferase reporter gene assay. These sequences were used to design DT-A expressing, therapeutic vectors (P3-DT-A and P4-DT-A). The activity of both was determined in cell lines (*in vitro*) and the activity of P3-DT-A was determined in a heterotopic animal model (*in vivo*). The treated cell lines highly responded to the treatment in a dose-response manner, and the growth rate of the developed tumors *in vivo* was highly inhibited (70%) after intratumoraly injection with P3-DT-A compared to non-treated tumors ($P < 0.0002$) or tumors treated by luciferase gene expressing LucP3 vector ($P < 0.002$).

Key Words: insulin like growth factor 2, IGF2, bladder cancer, gene therapy, diphtheria toxin, regulatory sequences

INTRODUCTION

Bladder carcinoma is one of the most common urologic malignancies. 93% of the bladder cancers are transitional cell carcinoma (TCC) [1]. Different treatments including Bacillus Calmet-Guerin (BCG) and transurethral resections of the bladder tumor (TUR-BT) are used to cure TCC, however high recurrence rates are seen [1]. Different strategies have been proposed for targeted cell killing of cancer tissues including the exogenous delivery of a suicide gene under the control of regulatory sequences of genes differentially expressed in tumors [2,3]. The Diphtheria toxin A-chain (DT-A) was introduced as a candidate for cancer cell killing [4–7]. DT-A peptide catalytically ADP-ribosylates the diphthamide residue of the cellular elongation factor 2 (eEF-2), inhibiting protein synthesis leading to cell death [8,9]. Unintended toxicity to other cells can be avoided by introducing the DT-A gene under the control of regulatory sequences of genes differentially expressed in tumors. Moreover, DT-A released from the lysed cells is not able to enter the neighboring cells in the absence of the DT-B-chain [5].

The 67-aa IGF2 is a member of the insulin like growth factor family that is involved in cell proliferation and differentiation [10]. The human IGF2 gene contains 9 exons (E1–9) and 8 introns [10,11], and is transcribed from 4 different promoters (P1–P4) producing 4 different transcripts [12–14]. All four transcripts share a common coding region and a common 3.9kb 3′-UTR, but variable 5′-UTRs [11]. IGF2 is an imprinted gene that is almost exclusively expressed from the paternal allele [15–17]. The P3 and P4 promoters are the major IGF2 promoters during embryogenesis and tumor development, while P1 is exclusively active in adult liver tissue and P2 activity is rarely detected in adult human tissue [10]. Increased expression of IGF2 as a result of the loss of its imprinting is frequently seen in a variety of human diseases and tumors [17–20]. In addition, abnormal signal transduction and/or promoter activation was reported as a major mechanism for the IGF2 overexpression in a variety of tumors including bladder carcinoma, hepatocellular carcinoma, breast cancer and prostate cancer [21–25].

The IGF2 regulatory sequences might be good candi-

1525-0016/03 $30.00

doi:10.1016/S1525-0016(03)00056-X

dates for targeted cancer cell killing including TCC, as it can be administered to patients with high IGF2 expressing cancer cells (patient-oriented gene therapy). Here we report high levels of IGF2 P3 and P4 driven mRNA expression in TCC. Therefore we have constructed expression vectors carrying the diphtheria toxin A-chain gene, under the control of the P3 and P4 regulatory sequences. In this communication we show that these constructs are able to selectively kill tumor cell lines and inhibit tumor growth both *in vitro* and *in vivo*. The cytotoxic activity of these constructs is in accordance to the endogenous IGF2 mRNA expression from P3 and P4 promoters and to the *in vitro* promoters activity assayed by the luciferase reporter gene.

RESULTS

The Level of IGF2 P3 and P4 Transcripts in Cell Lines

We determined the level of IGF2 expression driven by P3 and P4 promoters in the cell lines HepG2, T24P and Skhep1 (Fig. 1A). The HepG2 cell line showed the highest level of P3 transcript expression, while the T24P cell line showed medium P3 expression. The P4 transcript expression was nearly equal in both HepG2 and T24P cell lines. Skhep1 showed no P4 expression but relatively high P3 expression.

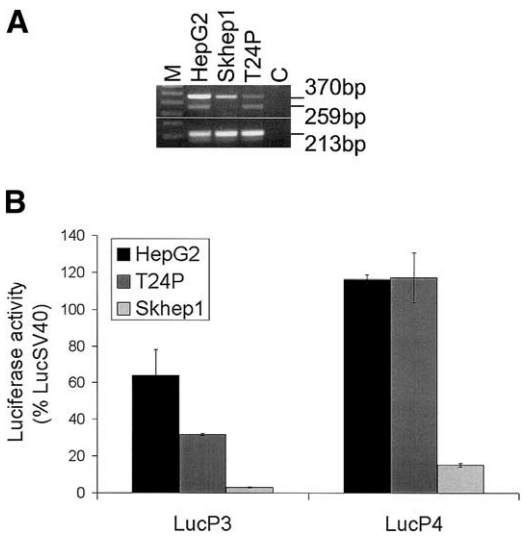

FIG. 1. The activity of IGF2 P3 and P4 promoters in HepG2, T24P and Skhep1. (A) The level of the endogenous P3 and P4 transcripts determined by RT-PCR (see Materials and Methods). (M) Is 100-bp ladder (Amersham Pharmacia Biotech Inc) and (C) is negative control. The sizes of the PCR products are 370, 259 and 213 bp for P3, P4 and Histone internal control respectively. (B) Each cell line was transfected with 3μg of LucP3, LucP4 or 5μg LucSV40. The luciferase activity of LucP3 and LucP4 was determined by the Luciferase Assay System kit (E-1500-Promega), and normalized to the activity of LucSV40 plasmid and the results are expressed as % of LucSV40 in the corresponding cell line.

TABLE 1: Endogenous P3 and P4 expression in normal bladder and TCC tissues determined by RT-PCR and ISH

	Determined by PCR		Determined by ISH
	Normal bladder	TCC	TCC
P3	0/4	5/10	11/19
P4	0/4	6/10	7/19

Fourteen samples were tested by PCR, among which 4 samples were normal bladder from independent patients. The results represented for ISH are the number of strongly positive samples (+2 to +3) out of the total number of samples analyzed.

The Level of IGF2 P3 and P4 Transcripts in Human Bladder Carcinoma Tissue

Different human bladder carcinoma and normal bladder tissue samples were tested for IGF2 expression from P3 and P4 promoters by PCR or by in situ hybridization (ISH) (Table 1). Five out of the 10 TCC samples examined by PCR showed both P3 and P4-driven IGF2 RNA expression and another sample expressed only the P4-driven transcript. The level of the transcripts varied from very high to low. However, none of the 4 normal bladder samples tested by PCR expressed IGF2 from either of P3 or P4 promoter. On the other hand 11 and 7 out of the 19 TCC samples examined by ISH showed high IGF2 expression from P3 and P4 promoters respectively, while 7 were negative for both of the transcripts (Table 1). The intensity of ISH staining was heterogeneous and varied from +1 to +3, and was mostly focal (Fig. 2). Two of the samples tested by ISH, that showed strong staining from both promoters, contained non-tumor mucosa adjacent to the tumor. One of them showed low staining from the two promoters in the surrounding normal-like tissue.

The Activity of P3 and P4 Regulatory Sequences in Cell Lines

The luciferase activity controlled by P3 and P4 (LucP3 and LucP4 respectively) was determined in the three human cell lines (HepG2, T24P and Skhep1). Figure 1B shows that the relative LucP3 activity in the different cell lines is (HepG2> T24P> Skhep1). The activity of LucP3 construct is in accordance with the endogenous expression of the P3 transcript in HepG2 and T24P but not in Skhep1 (Fig. 1A and B). LucP4 activity on the other hand was in accordance to the endogenous expression from P4 in the three-tested cell lines with equal expression in HepG2 and T24P and minimum expression in Skhep1 (Fig. 1A and B).

In Vitro DT-A Expression Under the Control of P3 and P4 Regulatory Sequences

P3 and P4 are able to drive the expression of the DT-A gene in the cell lines HepG2, T24P and Skhep1. Both P3-DT-A (Fig. 3A) and P4-DT-A (Fig. 3B) gave a dose-response behavior in the three cell lines. P3-DT-A was able to reduce the LucSV40 activity to about 50% at concentrations as low as (0.01 and 0.015 μg/well) in the HepG2

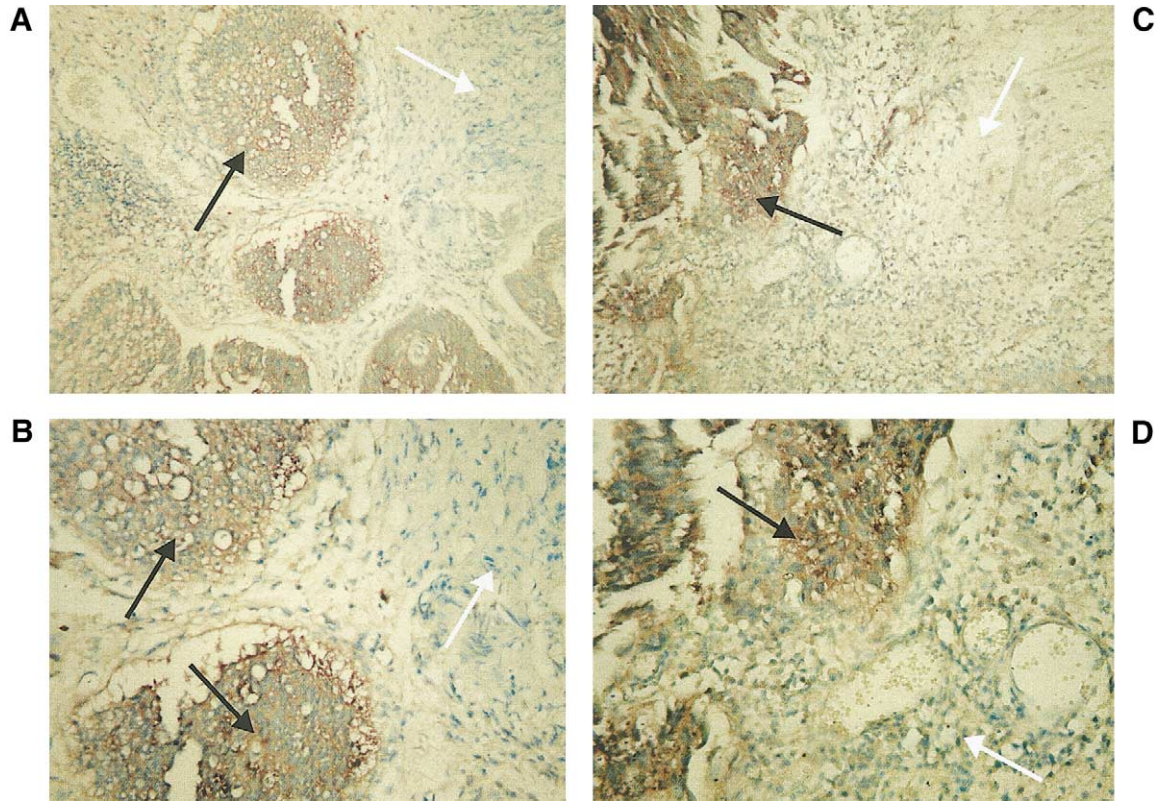

FIG. 2. *In situ* hybridization detection of the expression of P3 transcript (A and B) and P4 transcript (C and D) in TCC tissue samples. Black arrows indicate strong positive staining in the cytoplasm; white arrows indicate non-tumor bladder tissue. The nuclei are stained by Giemsa counter stain. Some positive signals are seen in the nuclei as the probes can hybridize to mRNA as well as to DNA. (Magnification of A and C is 20× and of B and D 40×).

and T24P cell lines respectively. Fifty percent inhibition by P4-DT-A was obtained at very low concentrations (<0.005 μg/well) in HepG2 and T24P cells. However, relatively high concentrations of P3-DT-A and P4-DT-A were needed to achieve 50% reduction in Skhep1.

Both the human P3 and P4 are active also in the murine MBT-2-t50 cell line, which we used to establish the animal model for bladder cancer described in "Materials and Methods". They show comparable activity to those of the relevant mouse P2 and P3 promoters. Moreover they both are able to drive the expression of the DT-A toxin, which reduces LucSV40 activity in this cell line by (79.5 ± 2.3% and 63.9 ± 3.7%) by 0.5μg/well of P3-DT-A and P4-DT-A respectively ($P < 0.003$ for P3-DT-A and $P < 0.007$ for P4-DT-A) (data not shown).

In Vivo Tumor Growth Inhibition by P3-Driven DT-A Expression

We chose the P3-regulated vector for further examination in order to evaluate its potential to drive DT-A expression *in vivo* using an animal model for bladder cancer as described in "Materials and Methods". Figure 4A shows that 3 injections of P3-DT-A in two days intervals were able to inhibit the tumor development by at least 70% compared to those of LucP3 ($P < 0.002$) and to the non-treated tumors ($P < 0.0002$). This effect emerges early after the first treatment of the tumors. No difference however was seen between the growth of LucP3 treated and the non-treated tumors three days after the third injection ($P > 0.9$) as both were progressively increasing in size at the same rate (Fig. 4A). The group of four healthy animals, which received seven subcutaneous injections with P3-DT-A didn't show any signs of "negative" toxicity after 24 days. They continue to increase in weight in parallel to the group of three healthy animals injected by LucP3. Moreover, the histopathological examination of the two groups didn't reveal any evidence for toxicity in any of their internal organs (liver, kidney, heart, spleen, pancreas and adrenals) (data not shown). We have also calculated the ex-vivo density of each tumor and saw no significant differences between the non-treated group and the LucP3 treated ($P > 0.3$) or the P3-DT-A treated group ($P > 0.2$). Moreover, the histological examination of the excised tumors revealed that the average necrosis area, determined by visual examination, in the tumors treated

doi:10.1016/S1525-0016(03)00056-X

with P3-DT-A (56% ± 15%) was higher than in the tumors treated by LucP3 (39% ± 18%) ($P < 0.05$). No difference however was seen between the non-treated tumors (39% ± 22%) and those treated with LucP3 (39% ± 18%) ($P > 0.95$). DT-A mRNA could be detected in the P3-DT-A treated tumors (Fig. 4B, lanes 3–6) analyzed by PCR but not in the LucP3 treated tumors (Fig. 4B, lanes 1, 2).

DISCUSSION

We applied a simple and convenient approach in our preliminary experiments for gene therapy. This approach involves the intratumoral injection of mouse with ectopically developed tumors by plasmid vectors expressing the DT-A toxin under the control of the IGF2 promoters P3 and P4. The LucP3 vector was used to transfect the tumor cells *in vivo* as a calcium phosphate co-precipitate, which enhances the transfection efficiency. The use of the plasmid vector avoids any immunological consequences resulting from successive injections by viral vectors. Moreover transient transfection of the tumor cells is sufficient for the toxin expression and cell killing. The *in vivo* model we used has the advantage of rapidly developing tumors, which allows short turn-around times for the experiments (3 weeks).

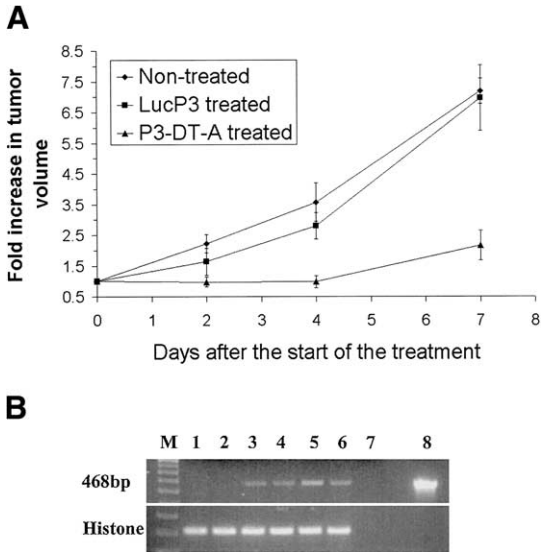

FIG. 4. Inhibition of tumor growth in response to P3-DT-A treatment. (A) The tumor sizes were determined prior to each treatment and before sacrifice. The folds increase in tumor volume was calculated relative to the initial volume at the day of the first treatment. (B) The DT-A mRNA expression was determined in some of the LucP3 and P3-DT-A treated tumors. 400 ng RNA (extracted from the tumors after sacrifice) were used for the RT-PCR reactions. Lanes 1 and 2: LucP3 treated tumors; lanes 3–6: P3-DT-A treated tumors. Lane 7: a blank control; lane 8: a positive control (50 ng P3-DT-A plasmid) and M is a 100-bp ladder. The upper panel is the 468 bp DT-A mRNA and the lower is the Histone internal control.

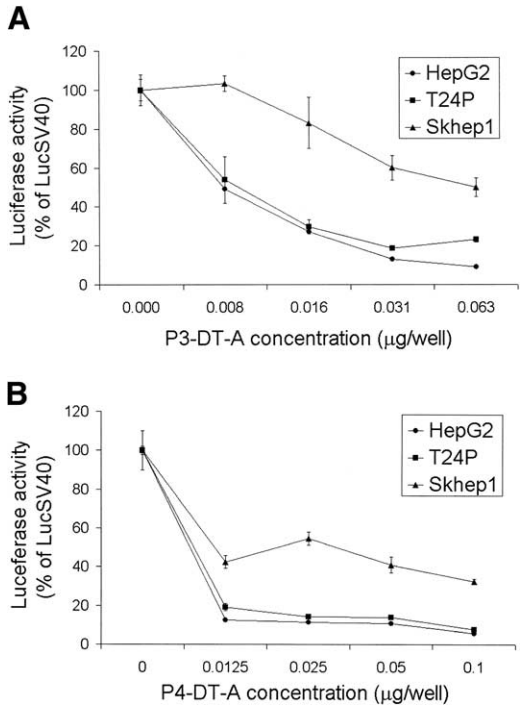

FIG. 3. Effect of the Therapeutic vectors P3-DT-A and P4-DT-A on HepG2, T24P and Skhep1 cells measured as reduction of LucSV40 activity. The cells were cotransfected with 5μg of LucSV40 and the indicated concentrations of P3-DT-A (A) or P4-DT-A (B). The decrease in LucSV40 activity was determined by comparison to the same cell type transfected only with LucSV40 as a measure for cytotoxicity.

In order to determine the validity of this approach for human TCC therapy, both RT-PCR and ISH techniques were applied to determine the level of IGF2 expression in TCC tissue samples from P3 and P4 promoters (Table 1). 16 out of 29 samples (55%) and 13 out of 29 (45%) showed high levels of expression from P3 and P4 respectively. All in all, 18 samples (62%) were found to express IGF2 from P3, P4 or both promoters and 11 samples (38%) didn't express the gene from any of the promoters. Moreover, none of the 4 independent normal samples analyzed by PCR showed IGF2 expression from any of the promoters. On the other hand, the low level of staining of the normal-like tissue adjacent to the tumor in one of the samples analyzed by ISH may be due to its entry into the early stage of transformation. This might have important implications for preventing tumor recurrence by gene therapy, as both the tumor and the premalignant tissues will be affected by the treatment. The results of (Table 1) are in accordance to previous reports that showed high levels of IGF2 mRNA and protein in 50% of bladder carcinoma tissue samples, which was localized in the malignant tissue [26,27]. This makes the IGF2 P3 and P4 regulatory sequences good candidates for the TCC targeted gene therapy. Moreover, the targeting of IGF2 expressing

cells in the tumor area is advantageous because IGF2 exerts its mitogenic effects in autocrine and paracrine manner [28,29] and contributes to tumor progression and proliferative activity [30]. Therefore, killing the IGF2 expressing cells not only will destroy the IGF2 expressing foci of the tumor but also will limit the IGF2 supply for other parts of the tumor in a bystander effect and thus may contribute to the inhibition of the tumor development.

Among the TCC samples analyzed by ISH, 7 samples showed expression from both promoters. Only two of those expressed the gene in the same localization from both promoters while the rest expressed the gene from each promoter in different tumoral location. This suggests that a combination of therapeutic vectors controlled by P3 and P4 regulatory sequences is expected to exert higher therapeutic potential.

We evaluated the correlation between the P3 and P4-regulated expression vectors in cell lines and the level of the endogenous IGF2 expression from P3 and P4 in these cell lines. The activity of the vectors was in general positively related to the endogenous expression levels of IGF2 from the relevant promoters except for LucP3 in Skhep1 (Fig. 1A and B). Moreover, the activity of the DT-A toxin expressing vectors (P3-DT-A and P4-DT-A) showed a dose response effect (Fig. 3A and 3B) and acted in accordance to their corresponding luciferase expressing vectors (LucP3 and LucP4) (Fig. 1B). The human P3-DT-A and P4-DT-A vectors were also active in the MBT-2-t50 murine cell line although higher concentrations are needed to reach the same inhibition as in the human cell lines. This can be explained by the extensive homology between the human and murine IGF2 genes and promoters' sequences [11]. Thus this cell line proved to be suitable for the generation of the animal model used later to examine P3-DT-A *in vivo*. P3-DT-A was able to highly inhibit the growth rate of the tumors induced in mice (Fig. 4A). At least 70% inhibition is obtained compared to LucP3 ($P < 0.002$) and to the non-treated tumors ($P < 0.0002$). This effect was detected early after the start of the treatment. The inhibition of tumor progression resulted exclusively from the toxic effect of the DT-A and not from the destruction of the tissue by the needle insertion and/or any possible effect of the plasmid backbone sequence. This is obvious as no significant difference was seen between the tumor sizes of the LucP3 treated and the non-treated groups ($P > 0.9$). Moreover, it is important to note that we didn't detect any signs of unwanted toxicity in the liver, kidney, heart spleen, pancreas and the adrenals of mice subcutaneously injected by P3-DT-A. We also introduced the calculated ex-vivo tumor density as a measure for the consistency of the tumors. No significant difference in the density was seen between the three groups. By this we exclude any possibility that the reduction in tumor size is a result of changes in the tumor consistency subsequent to the treatment. We also validated the tumor size calculations as a representative way for estimating tumor cell killing and thus the efficiency of the treatment. The cytotoxic effect of P3-DT-A was also obvious by the increase in cellular necrosis of the P3-DT-A treated tumors compared to the LucP3 treated and non-treated ones.

In this work we used the regulatory sequences of the IGF2 P3 and P4 promoters for two reasons. First, IGF2 gene is differentially expressed. We detected high levels of IGF2 expression from either the P3 or the P4 promoters in at least 62% of the TCC samples examined, but could not detect any IGF2 expression in four normal bladder tissue samples. Second, IGF2 plays a role in tumor development. By selective killing of cancer cells, which express IGF2, we deprive the neighboring tumor cells at least partly of their IGF2 supply.

At least a third of the TCC samples tested didn't express IGF2 from either P3 or P4 promoter, while the rest expressed IGF2 from both promoters in a heterogeneous manner. Therefore we propose a patient oriented approach to treat TCC. According to this approach the patient will be simultaneously treated with a combination of toxin expressing plasmids some of which are under the control of the IGF2 regulatory sequences, and other plasmids are under the control of the regulatory sequences of other differentially expressed genes. Moreover our proposed treatment may be applied in conjunction to other cancer therapy methods. This approach should be tested in appropriate animal models.

MATERIALS AND METHODS

Cell culture. The human bladder carcinoma cell line T24P and the hepatocellular carcinoma cell lines HepG2 and Skhep1 were obtained from the American Type Culture Collection (ATCC; Rockville, MD). They were chosen because they express varying levels of endogenous IGF2. The highly metastatic variant (MBT2-t50) of the MBT2 murine bladder carcinoma cell line was kindly provided by Dr. O. Medalia (Sackler Medical School, Tel-Aviv University, Tel-Aviv, Israel) and used to establish our working animal model because of their relatively fast tumor development and their ability to activate the human IGF2 promoters. The cells were grown as previously described [31].

RNA isolation and cDNA synthesis. Total RNA was extracted from cell lines or tissue blocks, using the RNA STAT-60™ Total RNA/mRNA isolation reagent (Tel-Test, Inc. Friendswood, TX), according to the manufacture's instructions. The RNA was treated by RNAse-free DNAse I (Roche Diagnostics GmbH, Mannheim) for eliminating any contaminating DNA.

The cDNA was synthesized from 2μg total RNA in 20 μl reaction volume by 10ng/μl of the oligo-$(dT)_{15}$ primer (Roche) and 10 units/μl M-MLV Reverse Transcriptase (GibcoBRL, United Kingdom) according to the manufacturer instructions. 2μl (about 200 ng) cDNA samples were taken for the amplification of the different transcripts using the described primers.

PCR amplification of P3 and P4 driven transcripts and DT-A coding mRNA. The PCR reactions were carried out in 25 μl volumes in the presence of 6 ng/μl of each of the forward and the reverse primers using 0.05 units/μl of Taq polymerase (TaKaRa Biomedicals, Japan) according to the kit instructions. The primer sequences used to amplify the IGF2 transcripts were (5′-GGACAATCAGACGAATTCTCC) for P3 transcript in the forward direction (from exon 5), (5′-CTTCTCCTGTGAAAGAGACTTC) for P4 transcript in the forward direction (from exon 6) and (5′-GCTT-

doi:10.1016/S1525-0016(03)00056-X

GCGGGCCTGCTGAAG) for both P3 and P4 transcripts in the reverse direction (from exon 7). The amplification conditions for P3 and P4 transcripts were 94°C for 5 min, followed by 28 cycles of 94°C for 1 min, 53°C for 40 sec and 72°C for 30 sec, and finally 72°C for 5 min. Different number of cycles was examined and we found that 28 cycles are low enough to keep the PCR in the linear phase. The primer sequences for DT-A amplification were (5′-TTCGTACCACGGGACTAAACCTGG) in the forward direction and (5′-CCACGTTTTCCACGGGTTTCAA) in the reverse direction. The amplification conditions for DT-A were 94°C for 5 min, followed by 31 cycles of 94°C for 1 min, 52°C for 40 sec and 72°C for 30 sec, and finally 72°C for 5 min. The integrity of the cDNA was assayed by PCR analysis of the ubiquitous, cell cycle independent, histone variant, H3.3 [32]. The products of the PCR reaction were run on 2% agarose in TAE electrophoresis running buffer (40 mM Tris acetate, and 2 mM EDTA, pH ~ 8.5), stained by ethidium bromide and visualized by UV.

DIG-labeled probe synthesis. We used a PCR strategy for generating template DNA for synthesis of labeled RNA probes. We designed forward primers that contain Sp6 promoter in their 5′-end and a reverse primer with T7 promoter incorporated in its 5′-end. The forward primers sequences were (5′-AGGGATCCATTTAGGTGACACTATAGAATCAGACGAATTC) for P3 and (5′-AGGGATCCATTTAGGAGACACTATAGAAAGAGACTTCCAG) for P4-specific transcripts. The reverse primer sequence was (5′-GGATCCTAATACGACTCACTATAGGGAGCCCATTGGTGT) for both P3 and P4 transcripts, which binds the first 11 bps of the 5′-end of exon 7, not sufficient to hybridize nonspecifically. The PCR conditions used to generate the T7/Sp6 templates were the same as described earlier for the synthesis of the P3 and P4 specific transcripts. The PCR products were purified from the gel by the GFX™ PCR, DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech Inc), sequenced and compared to the published sequences in the gene bank. 100 to 200 ng from the purified products were used as templates for the T7 and Sp6 polymerase (2 units/μl) (Roche) according to the manufacturer instructions in the presence of 2 units/μl RNAse inhibitor (Roche). T7 and Sp6 promoters were respectively used to drive the synthesis of the antisense and the control sense probes. The resulting probes were treated by 2 units of RNAse free DNAse I (Roche), pelleted and resuspended in appropriate volume of DEPC-treated double distilled water. The sizes of the synthesized probes were analyzed by running on 4% denaturing agarose minigel, and their labeling efficiency was determined by dot blot analysis.

In situ hybridization. Paraffin wax bladder carcinoma sections were kindly received from both Hadassah University Hospital and Makassed Hospitals (Jerusalem, Israel). The nonradioactive in situ hybridization washings and treatments were as described in [33]. Each section was hybridized by 30 μl of the hybridization solution containing 30–100 ng of the DIG-labeled probe at 52°C. The intensity of staining was indicated as (+1) for weak, (+2) for moderate and (+3) for strong signals. The degree of staining was referred to as focal (20%–70% of the cells) and defused (> 70% of the cells).

Plasmid construction. The human P3 and P4 regulatory sequences were subcloned from the Hup3 and Hup4 vectors described in [11] (a kind gift from Prof. P.E. Holthuizen, University of Utrecht, The Netherlands) into the pGL3 basic vector (LUC1) (Promega, Madison, WI), which lacks any eukaryotic promoter and enhancer sequences. The (LucP3) construct was prepared by digesting the P3 promoter (−747 to +140 relative to cap site) from (Hup3) plasmid by *Sac*I/*Sal*I and its insertion between the *Sac*I/*Xho*I restriction sites of the pGL3 basic vector. The (LucP4) construct was prepared by amplifying the P4 regulatory sequence (−645 to +123 relative to the cap site) from the (Hup4) vector using the primer (5′-ATAGGAGCTCATTCCCGGTCGGTCT) in the forward direction and (5′-ATACAAGCTTCGACTCTAGAGGATCC) in the reverse direction. The *Sac*I and *Hind*III sites were introduced to the 5′ end of the two primers respectively and used to clone the sequence at the same sites of the pGL3 basic vector. The DT-A containing vectors P3-DT-A and P4-DT-A were designed by replacing the luciferase gene in LucP3 and LucP4 respectively by the DT-A (prepared from the pIBI30-DT-A plasmid kindly donated by Dr. Ian Maxwell, University of Colorado, Denver, Co.) between the *Xba*I and *Nco*I restriction sites before or after the promoters cloning.

Transfection. A total of 0.3×10^6 cells were grown overnight in a six-well Nunc multidish (30mm). Transient transfection of the cells was performed using the calcium phosphate precipitation method [34]. In short, LucSV40 (Promega) and the other plasmids at the proper concentration were left to precipitate for 20 minutes at room temperature and used to transfect the cells for 16 hours. The medium was replaced by a fresh one and the cells were left to grow for another 48 hours. The cells were harvested and the luciferase activity was determined using the Luciferase Assay System kit (E-1500-Promega). The light output was measured using a Lumac Biocounter apparatus. The total protein content of the lysates was determined by the Bio-Rad (Hercules, CA) protein assay reagent, and the results were normalized to the total protein and expressed as Light units/μg protein.

LucSV40 was used as a positive control for the efficiency of transfection as it contains the SV40 promoter and enhancer, while LUC1 that lacks any regulatory sequences was used as a negative control to determine the basal nonspecific luciferase expression, which was found to be negligible. All experiments were done in triplicates and the results expressed as mean and standard error.

In vivo gene therapy. 0.33×10^6 MB-2-t50 bladder carcinoma cells (in 250 μl serum free DMEM medium) were subcutaneously injected into the dorsa of 6-7 weeks old C3H/He female mice (purchased from Harlan Laboratory, Jerusalem, Israel) as described in [3]. Measurable tumors appeared after 10 days. After 14 days of cell inoculation the developed tumors were measured by a caliper in two dimensions and subjected to different treatments. A group of 8 animals was treated with 50 μg/mouse of the plasmid containing the DT-A gene under the control of P3 promoter. Another group of 8 animals was treated by the injection of 50 μg of the luciferase expressing plasmid (LucP3). And a group of 4 animals was kept with no treatment. The plasmids were injected directly into the developed tumors as a calcium phosphate precipitate and the needle was left in the tumor for 30 sec to prevent back-diffusion. Each mouse of the treated groups received 3 injections of the proper plasmid in two days intervals. The dimensions of the tumor were measured before each injection and before sacrifice. The tumor volume was calculated by the formula ($0.5 \times L \times W$) where L is the longest and W is the shortest dimension. The animals were sacrificed 3 days after the last injection, the tumors were excised and their ex-vivo weight and volume were determined. Samples of the tumors were fixed in 4% buffered formaldehyde and processed for histological examination for evidence of necrosis and persistent tumor.

To detect any undesired toxicity of P3-DT-A, a group of 4 and a group of three control healthy animals were injected subcutaneous by 50 μg of P3-DT-A and LucP3 respectively every 2–4 days (a total of 7 injections). The animals' weight was recorded each time prior to the injection. The two groups were sacrificed after 24 days and their Liver, kidney, heart, spleen, pancreas and adrenals were taken and examined histopathologically for any signs for negative toxicity.

ACKNOWLEDGMENTS

This work was supported by the KERYX Biopharmaceuticals, Jerusalem, Israel.

RECEIVED FOR PUBLICATION JUNE 13, 2002; ACCEPTED FEBRUARY 7, 2003.

REFERENCES

1. Pow-Sang, J. M., and Seigne, J. D. (2000). Contemporary management of superficial bladder cancer. *Cancer Control* **7**(4): 335–339.
2. Abdul-Ghani, R., Ohana, P., Matouk, I., Ayesh, S., Ayesh, B., Laster, M., Bibi, O., Giladi, H., Molnar-Kimber, K., Sughayer, M. A., de Groot, N., and Hochberg, A. (2000). Use of transcriptional regulatory sequences of telomerase (hTER and hTERT) for selective killing of cancer cells. *Mol. Ther.* **2:** 539–544.
3. Ohana, P., Bibi, O., Matouk, I., Levy, C., Birman, T., Ariel, I., Schneider, T., Ayesh, S., Giladi, H., Laster, M., de Groot, N., and Hochberg, A. (2002). Use of H19 regulatory sequences for targeted gene therapy in cancer. *Int. J. Cancer* **98:** 645–650.
4. Lidor, Y. J., Lee, W. E., Nilson, J. H., Maxwell, I. H., Su, L. J., Brand, E., and Glode, L. M. (1997). In vitro expression of the diphtheria toxin A-chain gene under the control of human chorionic gonadotropin gene promoters as a means of directing toxicity to ovarian cancer cell lines. *Am. J. Obstet. Gynecol.* **177:** 579–585.
5. Maxwell, I. H., Glode, L. M., and Maxwell, F. (1992). Expression of diphtheria toxin

A-chain in mature B-cells: a potential approach to therapy of B-lymphoid malignancy. *Leuk. Lymphoma* **7:** 457–462.
6. Massuda, E. S., Dunphy, E. J., Redman, R. A., Schreiber, J. J., Nauta, L. E., Barr, F. G., Maxwell, I. H., and Cripe, T. P. (1997). Regulated expression of the diphtheria toxin A chain by a tumor-specific chimeric transcription factor results in selective toxicity for alveolar rhabdomyosarcoma cells. *Proc. Natl. Acad. Sci. USA* **94:** 14701–14706.
7. Maxwell, F., Maxwell, I. H., and Glode, L. M. (1987). Cloning, sequence determination, and expression in transfected cells of the coding sequence for the tox 176 attenuated diphtheria toxin A chain. *Mol. Cell Biol.* **7:** 1576–1579.
8. Choo, Y., Sanchez-Garcia, I., and Klug, A. (1994). In vivo repression by a site-specific DNA-binding protein designed against an oncogenic sequence. *Nature* **372:** 642–645.
9. Sandvig, K., and van Deurs, B. (1992). Toxin-induced cell lysis: protection by 3-methyladenine and cycloheximide. *Exp. Cell Res.* **200:** 253–262.
10. Engstrom, W., Shokrai, A., Otte, K., Granerus, M., Gessbo, A., Bierke, P., Madej, A., Sjolund, M., and Ward, A. (1998). Transcriptional regulation and biological significance of the insulin like growth factor II gene. *Cell Prolif.* **31:** 173–189.
11. Holthuizen, P. E., Cleutjens, C. B., Veenstra, G. J., van der Lee, F. M., Koonen-Reemst, A. M., and Sussenbach, J. S. (1993). Differential expression of the human, mouse and rat IGF-II genes. *Regul. Pept.* **48:** 77–89.
12. Pagter-Holthuizen, P., Jansen, M., van Schaik, F. M., van der, K. R., Oosterwijk, C., Van den Brande, J. L., and Sussenbach, J. S. (1987). The human insulin-like growth factor II gene contains two development-specific promoters. *FEBS Lett.* **214:** 259–264.
13. Pagter-Holthuizen, P., Jansen, M., van der Kammen, R. A., van Schaik, F. M., and Sussenbach, J. S. (1988). Differential expression of the human insulin-like growth factor II gene. Characterization of the IGF-II mRNAs and an mRNA encoding a putative IGF-II-associated protein. *Biochim. Biophys. Acta* **950:** 282–295.
14. Holthuizen, P., van der Lee, F. M., Ikejiri, K., Yamamoto, M., and Sussenbach, J. S. (1990). Identification and initial characterization of a fourth leader exon and promoter of the human IGF-II gene. *Biochim. Biophys. Acta* **1087:** 341–343.
15. Giannoukakis, N., Deal, C., Paquette, J., Goodyer, C. G., and Polychronakos, C. (1993). Parental genomic imprinting of the human IGF2 gene. *Nat. Genet.* **4:** 98–101.
16. Ekstrom, T. J., Cui, H., Li, X., and Ohlsson, R. (1995). Promoter-specific IGF2 imprinting status and its plasticity during human liver development. *Development* **121:** 309–316.
17. Ohlsson, R., and Franklin, G. (1995). Normal development and neoplasia: the imprinting connection. *Int. J. Dev. Biol.* **39:** 869–876.
18. Morison, I. M., and Reeve, A. E. (1998). Insulin-like growth factor 2 and overgrowth: molecular biology and clinical implications. *Mol. Med. Today* **4:** 110–115.
19. Wu, H. K., Squire, J. A., Catzavelos, C. G., and Weksberg, R. (1997). Relaxation of imprinting of human insulin-like growth factor II gene, IGF2, in sporadic breast carcinomas. *Biochem. Biophys. Res. Commun.* **235:** 123–129.
20. Ross, J. A., Schmidt, P. T., Perentesis, J. P., and Davies, S. M. (1999). Genomic imprinting of H19 and insulin-like growth factor-2 in pediatric germ cell tumors. *Cancer* **85:** 1389–1394.
21. Hahn, H., Wojnowski, L., Specht, K., Kappler, R., Calzada-Wack, J., Potter, D., Zimmer, A., Muller, U., Samson, E., Quintanilla-Martinez, L., and Zimmer, A. (2000). Patched target Igf2 is indispensable for the formation of medulloblastoma and rhabdomyosarcoma. *J. Biol. Chem.* **275:** 28341–28344.
22. Lee, Y. I., Lee, S., Das, G. C., Park, U. S., Park, S. M., and Lee, Y. I. (2000). Activation of the insulin-like growth factor II transcription by aflatoxin B1 induced p53 mutant 249 is caused by activation of transcription complexes; implications for a gain-of-function during the formation of hepatocellular carcinoma. *Oncogene* **19:** 3717–3726.
23. Bae, S. K., Bae, M. H., Ahn, M. Y., Son, M. J., Lee, Y. M., Bae, M. K., Lee, O. H., Park, B. C., and Kim, K. W. (1999). Egr-1 mediates transcriptional activation of IGF-II gene in response to hypoxia. *Cancer Res.* **59:** 5989–5994.
24. Mineo, R., Fichera, E., Liang, S. J., and Fujita-Yamaguchi, Y. (2000). Promoter usage for insulin-like growth factor-II in cancerous and benign human breast, prostate, and bladder tissues, and confirmation of a 10th exon. *Biochem. Biophys. Res. Commun.* **268**(3): 886–892.
25. Sohda, T., Yun, K., Iwata, K., Soejima, H., and Okumura, M. (1996). Increased expression of insulin-like growth factor 2 in hepatocellular carcinoma is primarily regulated at the transcriptional level. *Lab Invest* **75:** 307–311.
26. Li, S. L., Goko, H., Xu, Z. D., Kimura, G., Sun, Y., Kawachi, M. H., Wilson, T. G., Wilczynski, S., and Fujita-Yamaguchi, Y. (1998). Expression of insulin-like growth factor (IGF)-II in human prostate, breast, bladder, and paraganglioma tumors. *Cell Tissue Res.* **291:** 469–479.
27. Fichera, E., Liang, S., Xu, Z., Guo, N., Mineo, R., and Fujita-Yamaguchi, Y. (2000). A quantitative reverse transcription and polymerase chain reaction assay for human IGF-II allows direct comparison of IGF-II mRNA levels in cancerous breast, bladder, and prostate tissues. *Growth Horm. IGF. Res.* **10**(2): 61–70.
28. Rasmussen, A. A., and Cullen, K. J. (1998). Paracrine/autocrine regulation of breast cancer by the insulin-like growth factors. *Breast Cancer Res. Treat.* **47:** 219–233.
29. Kawamoto, K., Onodera, H., Kan, S., Kondo, S., and Imamura, M. (1999). Possible paracrine mechanism of insulin-like growth factor-2 in the development of liver metastases from colorectal carcinoma. *Cancer* **85:** 18–25.
30. Kawamoto, K., Onodera, H., Kondo, S., Kan, S., Ikeuchi, D., Maetani, S., and Imamura, M. (1998). Expression of insulin-like growth factor-2 can predict the prognosis of human colorectal cancer patients: correlation with tumor progression, proliferative activity and survival. *Oncology* **55:** 242–248.
31. Kopf, E., Bibi, O., Ayesh, S., Tykocinski, M., Vitner, K., Looijenga, L. H., de Groot, N., and Hochberg, A. (1998). The effect of retinoic acid on the activation of the human H19 promoter by a 3′ downstream region. *FEBS Lett.* **432:** 123–127.
32. Futscher, B. W., Blake, L. L., Gerlach, J. H., Grogan, T. M., and Dalton, W. S. (1993). Quantitative polymerase chain reaction analysis of mdr1 mRNA in multiple myeloma cell lines and clinical specimens. *Anal. Biochem.* **213:** 414–421.
33. Ariel, I., Miao, H. Q., Ji, X. R., Schneider, T., Roll, D., de Groot, N., Hochberg, A., and Ayesh, S. (1998). Imprinted H19 oncofetal RNA is a candidate tumour marker for hepatocellular carcinoma. *Mol. Pathol.* **51:** 21–25.
34. Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell Biol.* **2:** 1044–1051.