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(71) Applicant (*for all designated States except US*):
GEORGETOWN UNIVERSITY [US/US]; 37th & O Streets, N.W., Washington, District of Columbia 20057 (US).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **JUNG, Mira** [US/US]; 330 New Mark Esplanade, Rockville, Maryland 20850 (US). **DRITSCHLO, Anatoly** [US/US]; 8101 Fenway Road, Bethesda, Maryland 20817 (US).

(74) Agents: **BRUEHS, Martin, A.** et al.; Buchanan Ingersoll PC, PO Box 1404, Alexandria, Virginia 22313-1404 (US).

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(54) Title: RADIATION INDUCIBLE IEX-1 PROMOTER

(57) Abstract: DNA molecules, such as expression vectors, comprising a radiation-inducible IEX-1 promoter operably coupled to a sequence encoding a biologically-active protein or polypeptide are useful for gene transfer applications. Preferably, the promoter is an IEX-1 promoter that is modified to provide increased radiation response and or specificity. Methods for delivery of such a DNA expression vector to tissues of a host animal, and other methods of using such a molecule are described. The vectors and methods are useful for research purposes, for example, for the controlled modification and study of cellular response to radiation using in vitro and in vivo methods. The vectors and methods are also useful in, and relate to, therapeutic compositions and methods, for example in the treatment of cancers, such as prostate cancer.

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RADIATION INDUCIBLE IEX-1 PROMOTER

BACKGROUND

[0001] Despite gains in treatment success rates, improvements in treatment of
5 cancers continue to be needed. In many circumstances, trade-offs are required. For
example, where local tumor control is desired, potential gains in survival by
increasing radiation doses must be weighed against potential losses in quality of life.
Therefore, improvements in localized tumor control are a major therapeutic goal.
Moreover, improvements developed for the treatment of one form of localized cancer,
10 such as prostate cancer, can also be applied to the treatment other forms of cancer.

[0002] Cytokines, such as tumor necrosis factor (TNF), interleukins (ILs), and
interferons (IFs), have been used as agents for cancer therapy. TNF- α shows strong
synergistic action with interferon in *in vitro* cytotoxic effects. However, high-dose
delivery can lead to systemic toxicity, presumably through diffusion into the blood
15 stream or dose dumping. Expression of cytokines driven by gene transfer vectors can
be used to produce cytokines *in situ*. However, continuous *in situ* expression, i.e.,
driven by a constitutive promoter, may not be optimal, for example, in the absence of
spatial localization of expression of a cytokine.

[0003] Radiation-inducible expression vectors have been proposed to permit
20 controlled expression of therapeutic polypeptides in conjunction with radiation
treatment. Various methods of using gene transfer vectors containing radiation-
responsive promoters to treat tumors have been proposed. For example, U.S. Patent
No. 6,156,736, to Weichselbaum et al., discloses a method for inhibiting growth of a
tumor by delivering a DNA molecule to the tumor that comprises a radiation-
25 responsive enhancer-promoter comprising a distal CArG domain of an Egr-1
promoter, a TNF- α promoter, or a c-jun promoter linked to a region encoding a
protein such as cytosine deaminase; and, exposing the tumor to an expression-
inducing dose of ionizing radiation to express the protein, which inhibits the growth

of the tumor. U.S. Patent No. 5,571,797, to Ohno et al. discloses a method wherein a radionuclide in a dose effective to promote expression of the gene coupled to a radiation-responsive promoter is delivered to the host tissue.

[0004] Transcriptional regulation plays a significant role in the response of cells to ionizing radiation, including activation of DNA damage repair pathways, cell cycle arrest and/or apoptosis (1, 2). Genes induced by ionizing radiation have been characterized as early-, intermediate- and late-response genes based on the timing of induction (3, 4). Several genes, including those encoding for transcription factors NF- κ B, c-fos, c-jun and Egr-1, have been characterized as immediate early-response genes (5-7) and have been shown to be responsible for the subsequent induction of later-responding genes (8, 9). Of the immediate-early response genes characterized, only those encoding NF- κ B, c-fos, c-jun, Fra-1 and Egr-1 have been shown to be induced by ionizing radiation (10).

[0005] Datta et al. (11) showed an increase in the transcription of the EGR-1 gene upon treatment with radiation; Papathanasiou et al. (12) have identified a set of growth arrest and DNA damage-inducible (GADD) genes which are induced by X-rays and UV irradiation; and, Brach et al. (13) showed that treatment with ionizing radiation resulted in the activation and subsequent binding of NF- κ B to DNA. However, relatively few early-response genes have been described which are radiation-inducible.

SUMMARY

[0006] A DNA molecule comprising a radiation-inducible promoter, the IEX-1 promoter, and methods for using such a molecule are described herein. In preferred embodiments, the IEX-1 promoter is modified to delete certain regions not essential to radiation response. For example, the promoter can be operably coupled to a sequence encoding a biologically-active polypeptide. In an exemplary embodiment, a DNA molecule comprising a modified IEX-1 promoter has a portion of the promoter region

upstream of the CREB-like consensus sequence deleted and comprises nucleotides of the IEX-1 promoter including the region from about the CREB-like sequence to at least the TATA box and preferably including the transcription initiation site.

Alternatively, the IEX-1 promoter can be deleted of nucleotides upstream of about
5 nucleotide -603 and can comprise the portion of the IEX-1 promoter from about nucleotide -603 to nucleotide 1 of the IEX-1 gene. In another exemplary embodiment, a DNA molecule comprising a modified IEX-1 promoter has a portion of the promoter upstream of the keratinocyte enhancer consensus sequence region deleted and comprises nucleotides including from about the keratinocyte enhancer sequence to
10 about 35 residues downstream of the TATA box sequence. For example, in this embodiment, the promoter can be deleted of sequences upstream of about nucleotide -302 and can comprise from about nucleotide -302 to nucleotide 1 of the IEX-1 gene. In another exemplary embodiment, a DNA molecule comprises a modified IEX-1 promoter comprising the regions of the IEX-1 promoter from about nucleotide -603 to
15 about nucleotide -495 and the region from about -302 to nucleotide 1. In such an embodiment, the region of the IEX-1 promoter upstream of about nucleotide -603 or the region from about nucleotide -495 to about nucleotide -302 is deleted.

[0007] In another exemplary embodiment, a DNA sequence as described above can have the promoter operably coupled to a sequence encoding a biologically-
20 active polypeptide or protein. Suitable biologically-active polypeptides include, but are not limited to, a cytokine, an interferon, an interleukin, a tumor necrosis factor, a polypeptide hormone, insulin, growth factor, angiostatin, an antibody, antibody fragment, an immunogenic polypeptide, other active polypeptides, combinations thereof and the like. In another exemplary embodiment, the DNA molecule can be in
25 the form of a recombinant viral genome comprising a sequence as described above, or a recombinant retroviral genome encoding a DNA sequence as described above.

[0008] An exemplary method of introducing a radiation-inducible gene of interest into a host cell comprises, providing a DNA molecule comprising a modified IEX-1 promoter, wherein the promoter sequence is operably coupled to a coding

sequence for the gene of interest, and causing the DNA molecule to be transferred into a host cell. Preferably, the step of causing the DNA to be transferred into a host cell comprises exposing the cell to naked DNA or to DNA in the presence of one or more compounds that are known to promote transfection.

5 [0009] In another exemplary embodiment, the sequence of such a DNA can be encoded in the genome of an infectious viral particle and the DNA sequence can be transferred into a host cell by exposing the cell to the viral particle. In another exemplary embodiment, the transcription of the transferred gene is induced by exposing the cell to an effective dose of radiation. In another exemplary embodiment, the host cells are tumor cells. In another exemplary embodiment, the gene of interest encodes a therapeutic polypeptide. In another exemplary embodiment, the gene of interest encodes a marker polypeptide. In another exemplary embodiment, the method can comprise loading a composition comprising the DNA molecule into a vector transfer implant and placing the vector transfer implant within a selected site in the body of a host organism.

[0010] An exemplary method of treating tumors comprises, providing a composition comprising a DNA molecule comprising an IEX-1 promoter operably coupled to a sequence encoding a therapeutic polypeptide or protein; introducing the composition into the vicinity of a tumor; and, exposing the tumor to a dose of radiation sufficient to cause expression of the therapeutic polypeptide or protein. In one embodiment of the method, introduction of the composition into the vicinity of the tumor is accomplished by steps including loading the composition into a vector transfer implant and placing the vector transfer implant into the tissue of the tumor. In an alternative embodiment, introduction of the composition into the vicinity of a tumor can involve direct local application, infusion, or injection of the composition, or targeted delivery methods such as the use of targeted liposomes or targeted virus vectors.

[0011] In other exemplary embodiments, the therapeutic polypeptide or protein can be a polypeptide or protein that causes radiosensitization of tissue or has a

cytotoxic effect. In another exemplary embodiment, the therapeutic polypeptide or protein can be a cytokine. For example, the therapeutic polypeptide or protein can be chosen from among a TNF, an interleukin, an interferon, combinations thereof and the like. In another exemplary embodiment, the radiation dose can be the maximum dose appropriate for radiotherapy of the affected tissue. In another exemplary embodiment, there is a synergistic effect of the radiation-induced expression of the therapeutic protein or polypeptide together with the radiation dose. Alternatively, in exemplary embodiments where there is a synergistic effect, the radiation dose can be lower than the usual dose appropriate for radiotherapy of the affected tissue.

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BRIEF DESCRIPTION OF THE DRAWINGS

[0012] FIG 1: Illustrates a schematic of an exemplary expression vector in which a 0.6 kb fragment of the IEX-1 promoter (Kpn1-Sal1), which includes the CREB-like binding sequence, is inserted into the EcoRV site of a pcDNA3 vector from which the CMV promoter has been removed. The modified IEX-1 promoter is linked to a 0.6 kb HindIII fragment of cDNA encoding mature TNF- α

15

[0013] FIG 2: Shows the nucleotide sequence of the IEX-1 promoter and intron. Consensus sequences for features such as transcription factor binding sites, the TATA box, transcription initiation site for the IEX-1 gene (+1), and start codon (M) are labeled.

20

[0014] FIG 3: Map of exemplary IEX-1 promoter fragments generated by PCR. Numbers correspond to nucleotides at the beginning and end of fragments relative to the transcription start site (marked +1 in FIG. 2). Transcription factor consensus sequences of interest contained in each fragment are labeled.

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Transcriptional activation of the deletion modified IEX-1 promoters (IEXp1, 2, 3, 4, 5, and 6) as detected by luciferase reporter gene expression is indicated.

DETAILED DESCRIPTION

[0015] We have cloned and characterized the IEX-1 promoter and found that the IEX-1 promoter is well suited for gene transfer applications where radiation induction of gene expression is desired. Expression from the IEX-1 promoter can be induced by radiation. For example, an about 3 fold to about 4 fold increase in expression can be realized following exposure to about 10 Gy of ionizing radiation. Exposure to such doses is clinically feasible if 3-D conformal, intensity modulated radiotherapy, or radiosurgical techniques are employed. Such a promoter has utility, for example, in researching, *in vivo*, the effect of polypeptides of interest when expressed in radiation-exposed cells, and in the treatment of tumors in conjunction with radiation therapy.

[0016] DNA molecules comprising an IEX-1 promoter, preferably an IEX-1 promoter modified to increase radiation response and/or specificity, preferably coupled to a heterologous gene sequence encoding a biologically-active protein or polypeptide of interest can be used. The biologically-active polypeptide can be an indicator polypeptide, such as luciferase, an experimental polypeptide, such as for the study of modified radiation responses, or a therapeutic polypeptide, such as a cytokine or other therapeutic polypeptide. Where the application calls for increasing radiation sensitivity of host cells, preferred polypeptides of interest include cytokines, such as, for example, TNF- α .

[0017] The immediate-early induction of the human IEX-1 gene in response to ionizing radiation has been described (14). We have now analyzed the 5' flanking region of the IEX-1 genomic DNA. Figure 2 shows the sequence of the IEX-1 promoter. Unless otherwise indicated, nucleotide positions of elements of the promoter are given relative to the translation initiation start site, labeled (+1) at position 1463 of Figure 2.

[0018] Using DNA walking an about a 1462 bp long IEX-1 promoter was isolated. By computer-assisted analysis (FINDPATTERNS program, GCG Sequence

Analysis Software), we find that the IEX-1 promoter contains numerous consensus transcription factor recognition sites as well as an alu repeat (about nucleotide -303 to about nucleotide -551). The transcription initiation site was determined by primer extension. A TATA box is localized at about position -35 (i.e. 35 bases upstream of the transcription start labeled +1 in FIG 2). In addition, a number of transcription factor binding sites have been identified, for example: CAAT (nucleotide -52); AP4 (nucleotide -85); core enhancers (nucleotide -1453, -1206 and -1030); silencer (nucleotide -479) (15); SIF (nucleotide -488, within an alu repeat)(16); T-antigen binding site (nucleotide -671 and 71, within the intron); SP-1 (nucleotide -56 and -663); INF (nucleotide -272) (17); 3 NF- κ B-like sites (nucleotide -270, -128 and -815); and, a CREB-like site (nucleotide -599) (18).

[0019] In addition to radiation, expression of the native IEX-1 gene can be stimulated by serum, protein kinase C activators, and, to some extent, by UV-light. By modification of the promoter, such as deletion or mutation of portions of the promoter that are not radiation-specific, it is possible to maintain or improve the radiation response while eliminating regions of the promoter that can respond to other activators.

[0020] To identify radiation-inducible regulatory elements in the IEX-1 promoter, a series of deleted fragments were generated and ligated to a luciferase reporter gene in a pCEP4 expression vector. The constructs were transiently transfected into the human squamous carcinoma cell line SCC-35. By assessment of nucleotide of luciferase reporter gene expression, we found that the construct, which encompasses an about 725 nucleotide promoter region, does not respond strongly to radiation treatment. In contrast, expression in SCC-35 cells transfected with the IEXp3 construct, which includes the CREB-like sequence and downstream nucleotides, was activated by radiation. Deletion down to nucleotide -495 (IEXp4) resulted in a decrease in radiation-induced expression relative to cells transfected with IEXp3. The region between about nucleotide -488 and about -472 contains consensus

recognition sequences for SIF (16) and a silencer (15), which may explain the decrease in transcriptional activity observed for cells with the IEXp4 construct.

[0021] Deletion to nucleotide -302 (IEXp5) of the SIF/silencer region produced radiation-induced transcriptional activity of the IEX-1 promoter at the highest level (about 3-fold) observed. The region between nucleotide -302 and -277 contained within the IEXp5 construct corresponds to a consensus sequence for the keratinocyte enhancer, which may explain the high transcriptional activity observed. A construct beginning at about nucleotide -277 (IEXp6) possessed almost no activity.

[0022] Thus, it can be desirable to delete portions of the IEX-1 promoter to make modified promoter constructs having greater radiation-specific response. A construct comprising a modified IEX-1 promoter that has a substantial portion of the promoter region deleted, but retains nucleotides including the CREB-like sequence and downstream sequence including at least the TATA box, such as a construct comprising from about nucleotide -603 to about nucleotide 1 of the IEX-1 promoter, is a preferred embodiment nucleotide of the promoter. Such a construct can have enhanced radiation response and specificity.

[0023] Alternatively, a construct comprising a modified IEX-1 promoter that has a substantial portion of the promoter region deleted, but retains nucleotides including from about the keratinocyte enhancer sequence to at least the TATA box or at least about 35 residues past the TATA box, such as a construct comprising from about nucleotide -302 to about nucleotide 1 of the IEX-1 gene, can have strong radiation response and improved specificity. Based on the foregoing, it is also possible to create a further modified IEX-1 promoter comprising the region from about nucleotide -603 to about nucleotide -495 spliced to the region from about nucleotide -302 to about nucleotide 1. Alternatively, residues in the regions containing consensus SIF and silencer sequences can be mutated or deleted from a construct containing sequence of the IEX-1 promoter including from the CREB-like sequence to the transcription start site.

[0024] The skilled practitioner will recognize that the precise boundaries of the cut and spliced sites in these constructs can be modified for her convenience in accordance with conventional practice in the art to make essentially equivalent constructs. For example, nucleotides near the boundaries can be mutated or inserted
5 to provide restriction endonuclease cut sites. The creation of such variations will be guided by the general understanding of transcription promoters known to the skilled practitioner, general principles of molecular biology, and an understanding of the location of functional sequence elements of the promoter, such as disclosed herein.

[0025] Recognizable consensus elements of the promoter that are not
10 intentionally deleted or mutated are preferably preserved. Modifications can be screened for any effects on function using methods that are described herein. Thus, it will be recognized that substantially equivalent constructs can be made with reference to the information disclosed in this application that are not identical to the nucleotide-by-nucleotide sequence shown in Figure 2. Rather, such sequences can be defined and
15 recognized as modified IEX-1 promoters by the combination, in sequence, of building blocks of recognizable functional sequence elements of the IEX-1 promoter, such as the consensus sequences of functional elements identified in Figure 2.

[0026] As one preferred example, a modified IEX-1 promoter can be constructed comprising the region of the IEX-1 promoter from about nucleotide -603
20 to about nucleotide -495 spliced to the region from about nucleotide -302 to about nucleotide 1. Elements upstream of the CREB-like sequence can be mutated, or more preferably deleted, the CREB-like consensus sequence at about nucleotide -603 and functional sequences immediately downstream to about nucleotide -495 are retained, the region containing the SIF and silencer regions are excised or rendered non-
25 functional by mutation, and the region from the keratinocyte enhancer to about nucleotide 1 is preserved.

[0027] An IEX-1 promoter, preferably a modified IEX-1 promoter as described above, can be used to provide radiation inducibility of a transferred gene. Accordingly, a particularly preferred use of the promoter is construction of a

recombinant DNA molecule comprising all or part of the IEX-1 promoter sequence, preferably a modified IEX-1 promoter. A preferred embodiment is a DNA molecule comprising a modified IEX-1 promoter sequence, wherein the promoter sequence does not contain any residues upstream of about residue -603. In alternative preferred forms, a DNA molecule is provided comprising a modified IEX-1 promoter sequence, wherein the promoter sequence does not contain any whole transcription factor recognition sequences upstream of the CREB-like sequence at about -599. In another preferred embodiment, a DNA molecule is provided comprising a modified IEX-1 promoter sequence, wherein the promoter sequence does not contain any whole transcription factor recognition sequences upstream of the enhancer (Enh) sequence at about -302, for example, comprising from about nucleotide -302 to about nucleotide 1 and no further upstream residues of the IEX-1 promoter. In yet another preferred embodiment, a DNA molecule is provided comprising a modified IEX-1 promoter sequence, wherein the promoter sequence does not contain any whole transcription factor recognition sequences upstream of the CREB-like sequence at about nucleotide -599 and wherein the region from about nucleotide -495 to about nucleotide -302 has been deleted, and comprising from about nucleotide -302 to about nucleotide 1.

[0028] The above DNA molecules preferably further comprise a coding sequence for a heterologous gene of interest operably coupled to the IEX-1 promoter sequence. The DNA molecule can be in the embodiment of a plasmid, linearized DNA. Alternatively, the sequence of such a DNA molecule can be encoded on a viral genome. In a preferred embodiment, the DNA molecule is a plasmid DNA comprising the minimum component sequences necessary to replicate the plasmid and the IEX-1 promoter coupled to a gene of interest. The DNA molecule may also comprise a sequence encoding a selection marker gene, such as an antibiotic resistance gene.

[0029] A method of introducing a radiation-inducible gene of interest into a host cell can be preformed using a DNA molecule as described above, the method can comprise providing a DNA molecule comprising an IEX-1 promoter sequence, such

- as a DNA molecule comprising a modified IEX-1 promoter as described above, wherein the promoter sequence is operably coupled to a coding sequence for the gene of interest, and causing the DNA molecule to be transferred into a host cell. Causing the DNA to be transferred into a host cell can comprise exposing the cell to naked
- 5 DNA, exposing the cell to the DNA in the presence of one or more compounds that are known to promote transfection, such as cationic lipids, products such as lipofectamine, and the like, or the DNA sequence can be encoded in the genome of an infectious viral particle, for example as an adenovirus (e.g. Ad5 or Ad2), a retrovirus or any suitable infectious gene transfer vector.
- 10 [0030] In a further aspect of this method, transcription of the transferred gene is induced by exposing the cell to an effective dose of radiation, particularly ionizing radiation. The host cell can be one of a plurality of similarly treated host cells. The host cell may be maintained *in vitro* such as in a liquid or plated culture or be *in vivo* such as endogenous or transplanted cells in an animal such as a vertebrate animal
- 15 including rodents, equines, bovines, avians, swine, canines, felines, and primates, including humans. In a highly preferred embodiment, the host cells may be in a tumor, for example the host cells may be hyperproliferative or neoplastic cells and/or cells immediately proximate to tumor or cancerous cells.
- [0031] The DNA molecules described herein can be used where it is desired to
- 20 induce the expression of a biologically-active protein or polypeptide in conjunction with radiation exposure, for example in a combination of gene and radiation therapy. Both radiation and gene therapy are used in the treatment of cancer. Ionizing radiation can be administered from outside the body using techniques to limit the exposure of surrounding tissue or administered locally by implanting a radiation source.
- 25 Recombinant cytokines have been attractive agents for cancer treatment. Gene therapy provides a means of producing a therapeutic compound, such as, for example, a cytokine, *in situ* in the diseased host. Radiation and gene therapy can be used concurrently to kill tumor cells via independent mechanisms, thereby minimizing the evolution of treatment-resistant tumor cells. For example, TNF- α enhances the

tumoricidal activity of ionizing radiation *in vitro* and *in vivo*. The killing action of TNF- α is proposed to occur following production of superoxide and hydroxyl radicals, which mediate oxidative damage. The combination of TNF- α and radiation has produced encouraging preliminary results. However, toxicity can result from systemic
5 delivery of TNF- α in fever, including nausea, loss of appetite, fatigue, and hypotension.

[0032] By creating a localized radiosensitization effect, enhanced local tumor control can be achieved in radioresistant tumors. Radiosensitive tumors can be controlled with lower doses of radiation, reducing radiation damage to normal tissue
10 parenchyma surrounding the tumor. Since ionizing radiation can be confined to the tumor by conformal radiotherapy, controlling induction of gene expression by radiation, can provide highly localized gene expression to the radiation-treated region. In combination, radiation therapy and localized cytokine gene therapy using radiation induction of cytokine expression, has advantages of both spatial and temporal control,
15 thereby improving the local effectiveness while minimizing systemic effects.

[0033] To enhance tumor treatment and circumvent the problem of systemic toxicity associated with the combination of cytokine and radiation therapy, we have developed an approach using cytokine gene therapy where expression of the cytokine is controlled by ionizing radiation which can be locally targeted. This can result in
20 increased effectiveness and reduced systemic toxicity, since the high concentration of cytokine is regionally localized. In a particularly preferred embodiment, the gene of interest can be, for example, a sequence encoding a cytokine such as an interferon, an interleukin, or a tumor necrosis factor. A preferred cytokine for this purpose is TNF- α . However other cytokines, and indeed any therapeutic polypeptide, can be used in
25 the method.

[0034] The gene of interest may encode any biologically-active protein or polypeptide such as, for example, a polypeptide hormone, insulin, growth factors, angiostatin, combinations thereof, and the like. As further examples, the gene of interest may encode an antibody, an antibody fragment, or an immunogenic

polypeptide targeted for cell surface display by splicing the immunogenic sequence between a secretion signal and a transmembrane domain.

[0035] Non-therapeutic genes of interest can also be usefully transferred. It may be useful to introduce a DNA molecule encoding a gene of interest, under the control of a native or modified IEX-1 promoter, into a host for research or diagnostic purposes. For example, the exemplary materials and methods described herein can be used to assess the effectiveness of a of a gene of interest as a radiation sensitizer, or as a radioprotective compound, or to study cellular or host radiation responses in the presence of a gene of interest that is expressed in conjunction radiation exposure.

10 Methods of preparing a DNA molecule that encodes a polypeptide and of operably coupling that sequence with a promoter sequence are known in the art.

[0036] Transfer of the nucleotide sequence into host cells may be directed to tumor or cancerous cells in an organism or the vicinity of a tumor by the nature of the physical delivery arrangement, i.e., by local injection or release, or by targeted delivery methods such as by a surface modified liposome or targeted viral gene transfer arrangements which are known in the art.

15

[0037] Desirable physical distribution of therapeutic gene vectors can be achieved using interstitial delivery techniques. These methods can provide significant advantages for the treatment of localized tumors, such as in prostate cancer, in combination with radiation therapy. The combination of these delivery techniques with the radiation-inducible gene transfer constructs provides new effective therapeutic approaches to the use of radio-sensitization genes in conjunction with radiotherapy while reducing effects of the treatment outside of the treatment site.

20

[0038] A preferred method of localized delivery comprises placement of a vector transfer implant containing a composition comprising a DNA molecule described above, within a selected site in the body of a host organism. The vector transfer implant can be made of any suitable material, such as metal, or polymer, and can be adapted to hold about 1 μ l of plasmid (or virus) containing solution. Larger

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and smaller implants can be used in accordance with the desired volume and the size of the target tissue region.

[0039] A vector transfer implant can be any arrangement of biocompatible material that can hold and release a composition comprising the vector, for example an absorbent polymer bead, a vessel comprising a permeable membrane or a small open container, such as a tube open on one or both ends and/or having an opening in the side. Additional factors under the control of the practitioner include the concentration and composition of the DNA solution. For example, the DNA solution can contain thickening or stabilizing compounds that slow the rate of diffusion of the DNA molecule, such as, for example, lipids, sugars, starch, combinations thereof and the like. The DNA solution can also contain compounds that are known to facilitate transfer of a DNA molecule by host cell, such as, for example, cationic lipids, lipofectamine, combinations thereof, and the like.

[0040] An implant comprising a composition of the DNA molecules described in this application can be interstitially implanted using known techniques for brachytherapy implantation. For example, the carrier devices can be implanted using ultrasound guided techniques for transperineal radioactive implant placement as practiced in the treatment of cancers of the prostate, head, neck, breast, pancreas, and sarcomas. Accordingly, such a method can be preferably applied to the treatment of any cancer that can be treated by brachytherapy methods, including prostate, head, neck, breast, pancreas, and sarcomas.

[0041] Indeed, it is possible to combine the delivery of gene carrier devices as described above with delivery of radiation sources in a single operation. The gene delivery and radiation sources are preferably separate units, however a radiation source device can be adapted to deliver the DNA molecule and the radiation source in a single unit.

[0042] Thus, a useful method can include introducing a DNA molecule encoding a gene of interest operably coupled to an IEX-1 promoter, preferably a modified IEX-1 promoter such as described above, into tissue of a host by loading a

composition comprising the DNA molecule into a carrier device, such as a small implant and placing that carrier device in the tissue of the host.

[0043] Further, the combination of local delivery of a DNA molecule as described above and radiation therapy is a preferred method of using such molecules in a therapeutic application. Radiation therapy is an established treatment modality for the management of clinically localized cancers. The common methods for delivering radiation therapy include external beam treatment, brachytherapy and combinations of the two. Substantial recent advances in the technical aspects of radiation delivery to the prostate have permitted dose escalation, demonstrating improved tumor control at higher doses. Unfortunately, the presence of dose-limiting normal tissues, such as the bladder and rectum, limit the extent to which dose escalations may be safely employed. Taken together, these observations indicate that biologic approaches can provide advantages, which may be complementary to radiation therapy (e.g., radiotherapy sensitizers, or salvage treatment using viral gene therapy).

[0044] For example, the "inverse square" reduction in radiation with distance from a point source effects means that implantation of radioactive sources into the affected tissue delivers a highly localized radiation. This can be particularly advantageous, for example in treating prostate cancer. Current techniques involve the use of transrectal ultrasound guidance for placement of needle applicators into the prostate and subsequent introduction of radioactive sources (I-125 or Pd-103 "implants") into the prostate parenchyma in the desired distribution. Such procedures are common and are easily tolerated by patients with little disruption in lifestyle.

[0045] Using similar technical approaches, "brachytherapy" technology may be extended to deliver radiosensitizers, cytokines or viral vectors into cancerous prostate using vector transfer implants. Combining radiation therapy with gene therapy, as described here, can provide substantial advantages. Spatial and temporal control of gene therapy can be achieved by conforming radiotherapy to the inoculated tumor bed. Both radiation and gene therapy treat the disease locally, killing tumor

cells via independent mechanisms, thereby minimizing the evolution of treatment-resistant tumor cells at the onset of treatment. With radiosensitization, enhanced local tumor control may be achieved in radioresistant tumors, and radiosensitive tumors may be controlled with lower doses of radiation, thereby
5 reducing radiation damage to normal tissue parenchyma surrounding the tumor.

[0046] Cytokines, such as ILs, TNF, IFs, have been used as products of gene transfer to target tissues or tumors by viral vectors via intratumoral injection or intravenous viral delivery. Sensitization of cancer cells to ionizing radiation would permit more effective treatment of "radiation resistant" cancers. Failure to cure
10 malignant tumors in subsets of patients has been attributed to inherent tumor radioresistance. The combination therapies described herein can provide improvements in effectiveness without resort to undesirable increases in radiation or the systematic administration of biochemical therapeutic compounds.

[0047] Localized radiation sensitization of tumors has been a long unmet
15 need. Delivery of genes expressing cytokines or other therapeutic polypeptides directly and specifically into a tumor is a particularly attractive approach. The ability to deliver radiation sensitizing genes or cytokines, using interstitial techniques with which radiation oncologists have familiarity is expected to provide for rapid acceptance in the art, particularly for use in combination with radiation therapy of
20 locally advanced tumors.

[0048] Thus, a method of treating tumors, for example cancerous tumors, is also provided. The method can comprise, providing a composition comprising a DNA molecule comprising an IEX-1 promoter operably coupled to a sequence encoding a therapeutic polypeptide or protein, loading the composition into a vector transfer
25 implant, placing the vector transfer implant into the tissue of the tumor, and exposing the tumor to a dose of radiation sufficient to cause expression of the therapeutic polypeptide or protein. The therapeutic polypeptide or protein is preferably a polypeptide or protein that causes radiosensitization of tissue or has a cytotoxic effect, such as a cytokine, e.g., a TNF (such as TNF- α), an interleukin, an interferon,

combinations thereof, and the like. The radiation dose can be equivalent to the maximum dose appropriate for radiotherapy. Because of a synergistic effect of the radiation-induced expression of the therapeutic protein or polypeptide, however, preferred embodiments can include a reduced radiation dose.

5

EXAMPLES

A. General Methods

[0049] Cell Lines: PC3 cells are maintained in MEM containing 5% fetal calf serum at 37 °C in 5% CO₂ with penicillin and streptomycin added to all media, and are tested to ensure freedom from mycoplasma contamination.

[0050] Subcutaneous Tumor Model: The mice (6-to-7 week old male BALB/c nu/nu for human tumors) are anesthetized with an i.p. injection of a 0.25 - 0.30 ml solution consisting of 84% bacteriostatic saline, 10% sodium pentobarbital (1 mg/ml: Abbott Laboratories, Chicago, IL) and 6% ethyl alcohol or inhalation of 2-3 minimal alveolar concentration of methoxyflurane. Tumors are induced by s.c. injection of 5x10⁶ PC3 cells in 0.1 ml. Tumors are measured by external caliper to the 0.1mm, and volumes are calculated ($V=H \times L \times W$).

[0051] Implantation of vector transfer implants: The vector transfer implants are loaded with 1 µl of plasmid containing buffer or lipofectamine solution and frozen on dry ice or at -70 °C until ready to use. The frozen implant is loaded into an 18 gauge hollow stainless steel needle applicator (Best Inc. disposable needle designed for prostate brachytherapy). The long axis of the tumor is identified and the loaded implant is introduced percutaneously into the tumor center. The implant length is 0.3 cm and the tumor diameter is 0.5 to 1.0 cm.

[0052] *In vitro* and *in vivo* assays of tumor response to TNF-α expression: For *in vitro* studies, following transfection and clonal selection, cells are exposed to 10 to 20 Gy gamma radiation. Following freezing and thawing, cell lysates are assayed for TNF-α using a Quantikine TNF-α ELISA kit (R&D kit Systems, Minneapolis, MN). For *in vivo* studies, male nude mice bearing PC3 xenografts (100 mm³ volumes) are

implanted with vector transfer implants carrying pLEX-1- TNF- α plasmid DNA prior to a single or multiple dose of total 10 to 20 Gy. Mice are shielded with lead so that only tumors are irradiated. Non-irradiated tumors serve as controls. Tumors are excised for analysis. Homogenized samples are assayed for TNF- α using a Quantikine
5 TNF- α ELISA kit.

[0053] For *in vivo* tumor control assays, tumor diameters are measured 2 or 3 times weekly with calipers following implantation and irradiation as above. Irradiation of the animals is carried out using a Cs radiator at 150 kV and 30 mA using an AL filter. The dose rate is 143 cGy/min. The xenografts are irradiated with 10 Gy
10 followed 24 h later by an additional 10 Gy. Growth delay is used to assess the effects of TNF- α expression on the tumor growth rate. The control group mice is given either with a single treatment or multiple treatments, either i.v. or i.t.

[0054] *In vivo* assays of tumor response to TNF- α expression and radiation: Tumor growth delay and TCD50 (radiation dose needed to control 50% of irradiated
15 tumors) are used to determine the response of tumors to TNF- α expression and irradiation. Tumor regression and re-growth are followed until the tumors grow to 20 mm diameter, at which time the animals are killed. For the TCD50 assay, mice are checked for the presence of the tumor at the irradiated site at 4-5 day intervals for up to 100 days.

20 [0055] Effect of TNF- α expression on apoptosis: tumors are excised and fixed and embedded in paraffin was for histological sectioning, and then stained with hematoxylin and eosin. Apoptosis is determined by performing tunnel assay (Oncogene). Necrosis is also assessed by light microscopy.

[0056] Immunohistochemistry: Tumor tissues are fixed in 10% buffered
25 formalin (Fisher) and embedded in paraffin. Sections are mounted on poly-L-lysine coated slides. For detection of β -gal expression, slides are deparaffinized, permeabilized using tris-buffer (PH 7.5) containing 1% Triton 100, and incubated with blocking serum (normal goat) diluted 1:100 in PBS for 20 mm at room temperature. Slides are washed twice for 5 mm in PBS and incubated with primary

antibody for 1 h at room temperature. The primary antibodies, against β -galactosidase, are used according to manufacturer's instructions (Promega). Slides are rinsed with PBS and incubated with biotin-conjugated secondary antibody against IgG for 30 min at room temperature using the Streptavidin-AP system containing
5 biotinylated second antibody (KPL) and HistoMark kit (KPL) for color developing. Slides are then visualized by microscopy. For detection of GFP protein expression, slides are visualized using an epifluorescent microscope (Bx60 Universal Fluorescent Microscope; Olympus, Melville, NY) equipped with FITC filters and a camera (DKC 5000 Sony 3 CCD; Sony, New York, NY).

10

B. Determination of the Transcription Initiation Site of IEX-1 by Primer Extension.

[0057] Ten pmol of anti-sense primer corresponding to nucleotides 80-99 of IEX-1 cDNA was labeled by ^{32}P incorporation using T4 polynucleotide kinase as described by Kondratyev, A., Chung, K. & Jung, M., Cancer Res., 56:1498-1502
15 (1996) (14). The labeled primer was then added to 100 μg of total RNA isolated from SCC-35 cells. Reverse transcription was performed (final volume, 20 μl) at 42°C for 60 min and terminated by increasing the temperature to 95°C for 5 min. Aliquots (4 μl) of the reaction mixture were resolved on a 6% polyacrylamide sequencing gel. As a size marker for the transcription initiation site, IEX-1 genomic DNA was sequenced
20 using the same primer and then run in parallel with the primer extension sample.

C. Cloning of the IEX-1 promoter.

[0058] The 5' untranslated region (UTR) of the IEX-1 gene was cloned by using a PromoterFinder DNA walking kit (ClonTech). Human genomic DNA was
25 digested separately with five different restriction enzymes and then ligated to the PromoterFinder Adaptor provided by the manufacturer. The PromoterFinder libraries were then used as templates in two-round nested PCR reactions with IEX-specific primers (#1: 5'-AGTGCTGAGGTCCAGAGCGT-3'; and, #2: 5'-GTCCCGGGATGGTGGAGGGG-3') and adaptor primers. After the template

DNA was denatured at 80°C, the first round of PCR was performed with IEX-1 specific primer #1 and adaptor primer #1. The reaction conditions were as follows: 95°C, 1 min for 1 cycle; 95°C, 25 sec; 60°C, 1 min; 68°C, 4 min for 35 cycles; and, 8 min of extension at 68°C. The second PCR reaction was performed by using the first PCR product (1:10), IEX-1 specific primer #2 and adaptor primer #2 for 24 cycles. The PCR products were then analyzed on a 1.2% agarose gel.

[0059] The longest products (from DraI and SspI genomic libraries) were isolated and subcloned into a pCRII vector (Invitrogen) and sequenced. Two clones, corresponding to DraI (1361 bp) and Ssp I (1564 bp) PCR products containing the 5' UTR of the IEX-1 gene, were linked to a luciferase reporter gene in the pGL2 vector (Promega). For deletion constructs of the IEX-1 promoter, the specific regions of the IEX-1 promoter were generated by using PCR with primers modified with KpnI. The plasmid DNA was amplified in DH5a competent cells (GibCo-BRL).

15 D. Cell culture and Transfection.

[0060] The human head and neck squamous carcinoma cell line, SCC-35, was provided by Dr. R.R. Weichselbaum. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.4 mg/ml hydrocortizone.

20 [0061] Transient transfections were performed using lipofectin reagent (GibCo-BRL) and a pCEP4 expression vector containing a luciferase reporter gene driven by the IEX-1 promoter fragments (CAT2, 3, 4, and 5, Figure 3). SCC-35 cells at 50-60% confluence (10 cm² dishes) were washed twice with serum free media (SFM). DNA (2 µg IEX-1 and 5 µg green fluorescence protein as carrier) was premixed with 300 µl SFM and lipofectin reagent was premixed with 300 µl SFM for 45 min at room temperature. The DNA and lipofectin mixtures were then combined and incubated for an additional 15 min at room temperature. At this time, 2.4 ml of SFM was added to the DNA-lipofectin mixture and immediately overlaid onto SCC-35 cells. After an overnight incubation, the transfection was stopped by washing

of the cells with phosphate buffered saline (PBS, 2 times) and complete media (2 times). The cells were placed in complete media for approximately 8 h and then each 10 cm² dish split into 4 wells of a 6 well dish (60 mm²/well). The media was replaced with SFM approximately 18 h prior to irradiation (10 Gy, Mark Shephard irradiator) and cells harvested in reporter lysis buffer (Promega luciferase reporter gene expression kit) at the indicated times. Cells were lysed for 30 min on ice and then centrifuged for 20 min at 14,000xg (4°C). The cell extracts were then combined with luciferase reporter assay buffer and read in a luminometer. Three independent experiments were performed and the average relative luciferase activities were plotted.

E. Assessing distribution and efficacy of gene expression in a model system.

[0062] To assess gene delivery via implants into tumor, we used plasmid vectors as reporter markers, expressing p β -gal and pGFP (Clontech). β -galactosidase is a commonly used reporter molecule. Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is a unique reporter molecule that fluoresces bright green upon UV or blue light. GFP makes an excellent reporter because its fluorescence can be detected directly without additional cofactors or substrates. Whole organisms can be monitored as the reporter protein can be seen without fixation or disruption. It also has a half-life of greater than 24 hours. In fixed cells, fluorescence can still be detected after three months, based on manufacturer (Clontech) specifications.

[0063] Experiments were performed either by implanting implants loaded with plasmid or by injecting plasmid [10 μ g in PBS or lipfectamine (BRL)] into tumors. Each animal (n=5) tumor was implanted with reporter plasmid DNA (pGFP or β -gal). Implanted tumors were excised at 0, 24, 48 and 96 hrs. Tumors were divided into two sections parallel to the implanted implant. A half of the tumor was fixed and embedded in paraffin, while the other half was frozen in liquid nitrogen for enzymatic analysis. One piece of tissue section mounted on poly-L-lysine coated slides was stained using hematoxylin and eosin (H&E) to visualize adenocarcinoma. Others were

used for immunohistochemical analysis. Quantification of histological sections was obtained by analyzing images captured at 20x or 40x magnification to a computer.

F. Evaluation of cytokine gene delivery for radiation sensitization of tumor

5 [0064] Vectors expressing TNF- α cDNA under native and modified radiation responsive promoter (IEX-1) are used. The efficacy of cytokine expression via implant delivery is evaluated following radiation exposure on prostate tumor progression in a human xenograft model. Quantification and distribution of cytokine gene expression are analyzed by performing Western blot analyses, TNF- α detection by ELISA, tumor regression growth delay, apoptosis assays, and immunohistochemical assays.

10 [0065] The radiation inducible IEX-1 promoter is linked to the full-length TNF- α cDNA in the pcDNA3 plasmid (Invitrogen). The CMV promoter in pcDNA3 is used as a control. The whole native radiation-inducible IEX-1 promoter (1609 to +1 containing three NF- κ B binding sites) is spliced upstream to the full-length cDNA encoding TNF- α and is transcriptionally activated within the irradiated field to enhance radiation killing.

[0066] These constructs confer (i) radiation responsive and (ii) local and tissue-specific induced gene expression. Decreased systemic toxicity is expected because cytokine production is localized by radiation targeting, and the concentration leaking systemically should be small.

20 [0067] These vectors are first tested in cultured prostate cancer cells (PC3). Following stable transfection and clonal selection, about 5 clonal cells over-expressing TNF- α and one with vector (pcDNA3) itself will be subjected to test the biological consequences of TNF- α over-expression. Radiation clonogenic survival assays and apoptosis will be measured following treatment with ionizing radiation as described.

25 [0068] For *in vivo* tumor xenograft treatment, the construct is implanted via vector transfer implants into xenograft human prostate tumors in athymic nude mice and tumor growth delay is determined. Different expression levels of TNF- α is

achieved by activation of IEX-1 promoter following irradiation. Tumors are exposed to graded doses of gamma-irradiation using a ^{137}Cs source and the radiobiological response is analyzed by measuring the tumor growth delay. The regression in the relative tumor volume as a function of the increase in the intracellular TNF- α content in the implanted tumor is indicative of radiosensitization.

[0069] Tumor irradiation in 10 Gy fractions to a total dose of 20 Gy is performed. This dose is based on the tissue culture data which demonstrated a 3-4 fold increase in gene expression following 10 Gy radiation exposure. Quantification of TNF- α levels within xenografts are determined using immunohistochemical assays to show TNF- α levels within irradiated tumors as compared to sham irradiated tumors 15 min to 2 hours after initial treatment.

[0070] While the invention has been described in detail with reference to preferred forms thereof, it will be apparent to one skilled in the art that various changes can be made, and equivalents employed, without departing from the scope of the invention.

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WHAT IS CLAIMED IS:

1. A DNA molecule comprising a IEX-1 promoter modified such that a portion of the promoter region upstream of the CREB-like consensus sequence is deleted and that comprises a fragment of the IEX-1 promoter including from the
5 CREB-like sequence to the TATA box.
2. The DNA molecule of claim 1, wherein the IEX-1 promoter is deleted of nucleotides upstream of about nucleotide -603 and comprising the portion of the IEX-1 promoter from about nucleotide -603 to about nucleotide 1 of the IEX-1 gene.
10
3. A DNA molecule comprising a modified IEX-1 promoter that has a portion of the promoter upstream of the keratinocyte enhancer consensus sequence region deleted and comprises nucleotides including from about the keratinocyte enhancer sequence to the translation initiation start site.
15
4. The DNA molecule of claim 3, which is deleted in sequences upstream of about nucleotide -302 and comprising from about nucleotide -302 to about nucleotide 1 of the IEX-1 gene.
- 20 5. A DNA molecule comprising a modified IEX-1 promoter comprising the regions of the IEX-1 promoter from about nucleotide -603 to about nucleotide -495 and the region from about nucleotide -302 to about nucleotide 1 and not comprising the region of the IEX-1 promoter upstream of about nucleotide -603 or the region from about nucleotide -495 to about nucleotide -302.
25
6. A DNA molecule comprising the DNA sequence of any of claims 1 to 5 operably coupled to a sequence encoding a biologically active polypeptide or protein.

7. A DNA molecule according to claim 6, wherein the biologically active polypeptide is selected from the group consisting of among a cytokine, an interferon, an interleukin, a tumor necrosis factor, a polypeptide hormone, insulin, growth factor, angiostatin, an antibody, an antibody fragment, or an immunogenic polypeptide.
- 5
8. A recombinant viral genome comprising a sequence according to claim 6.
9. A recombinant retroviral genome encoding a DNA sequence according to claim 6.
- 10
10. A method of introducing a radiation-inducible gene of interest into a host cell, the method comprising, providing a DNA molecule comprising a modified IEX-1 promoter, wherein the promoter sequence is operably coupled to a coding sequence for the gene of interest, and causing the DNA molecule to be transferred into a host cell.
- 15
11. The method of claim 10, wherein causing the DNA to be transferred into a host cell comprises exposing the cell to naked DNA.
- 20
12. The method of claim 10, wherein causing the DNA to be transferred into a host cell comprises exposing the cell to the DNA in the presence of one or more compounds that are known to promote transfection.
- 25
13. The method of claim 10, wherein the DNA is encoded in the genome of an infectious viral particle and causing the DNA to be transferred into a host cell comprises exposing the cell to the viral particle.

14. The method of claim 10, wherein transcription of the transferred gene is induced by exposing the cell to an effective dose of radiation.

15. The method of claim 10, wherein the host cells are tumor cells.

5

16. The method of claim 10, wherein the gene of interest encodes a therapeutic polypeptide.

17. The method of claim 10, wherein the gene of interest encodes a marker polypeptide.

10

18. The method of claim 10, comprising loading a composition comprising the DNA molecule into a vector transfer implant and placing the vector transfer implant within a selected site in the body of a host organism.

15

19. A method of treating tumors, the method comprising, providing a composition comprising a DNA molecule comprising an IEX-1 promoter operably coupled to a sequence encoding a therapeutic polypeptide or protein; introducing the composition into the tissue of the tumor; and, exposing the tumor to a dose of radiation sufficient to cause expression of the therapeutic polypeptide or protein.

20

20. The method of claim 19, wherein the therapeutic polypeptide or protein is a polypeptide or protein that causes radiosensitization of tissue or has a cytotoxic effect.

25

21. The method of claim 19, wherein the therapeutic polypeptide or protein is a cytokine.

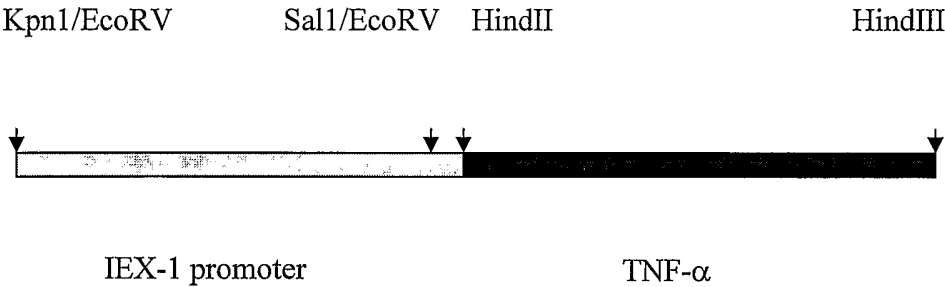
22. The method of claim 19, wherein the therapeutic polypeptide or protein is chosen from among a TNF, an interleukin, an interferon, and combinations thereof.

23. The method of claim 19, wherein the radiation dose is equivalent to
5 the maximum dose appropriate for radiotherapy of the affected tissue.

24. The method of claim 19, wherein the radiation dose is lower than the usual dose appropriate for radiotherapy of the affected tissue.

10 25. The method of claim 19, wherein there is a synergistic effect of the radiation-induced expression of the therapeutic protein or polypeptide together with the radiation dose.

FIGURE 1



enhancer

1 tattttaattg tgtattgttg ttgttgttgt tgttatttta tagtgacgag gtctcactat
histone H4 INF

61 gttgcccagg ctcctgagct caagtgatxx tcccacctta gccccccaaa atgctaggat
fos-like

121 tacaggcaag xxccaccatg ccccaccatt gtgtattact tttttaatct ttctatcctt

181 ccctccctcc ccaactttta ttttaaattc tggggatatat gtgcagggtt gttacctagg
enhancer

241 tatactgcat gatgctgagg tttggggtat gaatgatoct gtcaccocagg tactgagcat

301 agtacccaat agttagtggt tcaacctttg ccttactccc tccgtccttc ttctactggt

361 ctcaagttgc tattgttgcc atctttctgt ccatgagttc ccaatgtttg gttcccactt
INF enhancer histone H4

421 ataaagtgaga atgtgtgta tttggttttc tgttcctgag ttaatccgct taggataatg

481 gccaccagcc gcatccatgt tgctgcaaag cacataattt tgttcttttg tatggctacy
c-mos-like

541 cggtgtgtgt ctccatgtgt gctcaggcag gcaggtttgt gtctgtgtgt .gctagtgtgt
NFkB-like

601 tcattttctcc cttaatcttg actattttctg taacaatttc cttcttgggg agtttatatt

661 ctaaggatac ctacaagtgc atgaatgcgt gcatataact gggtcagtat tgcagcagga

721 tggcctgata tccaataaaa agccctcttg gcagggcacg gtggctcacg cctgtaatcc
T-antigen SP-1

781 cagcactttg ggaggccgag gaggggcaaac caaataccat acattctcac ttataagtgg
CREB-like

841 gaaccaaaaca ttgggaactc gtggacgtaa agatggcaac aatagcaact tgagactact
Ets-1

901 agaagaagga cggagggagc ggaggcagat cacgaggtca ggaaatcgag accaccttgg
SIF silencer SP1

961 ccaacagggt gaaaccccgt ctctactaaa aatacaaaaa ttagctggac gtgggtgcgc
Ets-1 Ap1

1021 gtgcctgtat tcccagctac tcagggaagct gaggcaggaa aatcgcttga accagggagt

1081 caggggttgc agtgagccga gatcggcct ctgcattcca gcctggcgac cgaacgagac
enhancer NFkB-like

1141 tgctccatct ccaaaaaaaaa aaaaaaggcc tgtgagggat cctgtggcta aagtgagccc
NFkB-like

1201 ctctccaggt gccacatgcc tcgacatgtg cctgcagccc gggatctcac ccacccccac
CRE-like

1261 tcacgactca cacactcaca acgtgcagtt gggcgccctag gattgtgcat gtcaagtctc
TATA Box NFkB-like AP4

1321 ~~caqccaatcc~~ ~~cttggtttaa~~ ~~ctgttgggaat~~ ttccagcccg ctgctgcaa ccgctcccca
SP-1 CAAT TATA Box
1381 gctg~~ggg~~gag gaggagttag aaggaccccgc ccaattttca ggagcacata aattacctct
+1 M
1441 gccggcagcc gacctcact tggccttaca ctccgctcgg ctcaccatgt gtcactctcg

FIGURE 2B

1501 cagctgccac cggaccatga ccacccctgca ggccccgacc cggccccct ccaccatccc
1561 gggaccccgg cggggctccg gtcctgagat cttcaccttc gaccctctcc cggagcccgc
1621 agcggccccct gccggcgcc ccagcgcttc tcgcgggcac cgaaagcgca gccgcagggt
Intron
1681 ctctaccctc gagtgtgag tatcgccgaa gtgggcattc gcggtgtgag ctgcctgga
1741 gtcactgggg aacgaccga ctccagagcc tcgacctgac ctgtctcctg tttgtctcc
1801 ccttagtccg gcgccagctg ccagtcgagg aaccgaacct agccaaaagg cttctctttc
1861 tgctgctcac catcgtcttc tgccagatcc tgatggctga agagggtgtg ccggcgcccc
1941 tgctccaga ggacgcccc aacgcgcgat ccctggcgcc caccctgtg tccccgtcc
2001 tcgagccctt taatctgact tcggagccct cggactacgc tctggacctc agcactttcc
2061 tccagcaaca cccggccgcc ttctaaactgt gactccccgc actccccaaa aagaatccga
2121 aaaaccacaa agaaacacca ggcgtacctg gtgcgcgaga gcgtatcccc aactgggact
2181 tccgaggcaa cttgaactca gaacactaca gcggagacgc caccggtgc ttgaggcggg
2241 accgaggcgc acagagaccg aggcgcatag agaccgaggc acagcccagc tgggctaggc
2301 cgggtgggaag gagagcgctg ttaatttatt tcttattgct cctaattaat atttatatgt
2361 atttatgtac gtcctcctag gtgatggaga tgtgtacgta atatttattt taacttatgc
2421 aagggtgtga gatgttccct ctgctgtaaa tgcaggtctc ttggtattta ttgagctttg
2481 tgggactggt ggaagcagga cacctggaac tgcggcaaag taggagaaga aatggggagg
2541 actcgggtgg gggaggacgt cccggctggg atgaagtctg gtggtgggtc gtaagtttag
2601 gaggtgactg catcctccag catctcaact ccgtctgtct actgtgtgag acttcggcgg
2661 accattagga atgagatccg tgagatcctt ccactttctt gaagtgcct ttaggggtgc
2721 tgcgaggtag agggttgggg gttggtgggc tgtcacggag cgactgtcga gatgcctag
2781 tatgttctgt gaacacaaat aaaattgatt tac

FIGURE 3

IEX-1 Promoter Constructs

