Heat-activated Transgene Expression from Adenovirus Vectors Infected into Human Prostate Cancer Cells
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ABSTRACT

Replication-deficient adenovirus expression vectors were used to introduce a recombinant DNA construct containing enhanced green fluorescent protein (EGFP) under control of a truncated, human heat shock promoter into human prostate cancer cells growing either exponentially or in plateau phase. This was done to measure controlled, heat shock-induced EGFP expression under conditions relevant to treating human cancers with heat-activated gene therapy. Both the temporal duration and magnitude of EGFP expression increased proportionately with stronger heat shocks (time at temperature) up to maximum values that were induced by 4 h at 41.0°C or 2 h at 42.0°C. Longer heat shocks at either temperature yielded no additional EGFP expression and ultimately reduced it. Maximal EGFP expression was induced in exponential cultures by heat shocks delivered 12–24 h after virus infection. Induction at progressively later postinfection times induced increasingly lower, peak EGFP expression. Maximal EGFP expression could not be induced until 48 h after infection of plateau phase cultures but could still be induced 180 h after virus infection. However, peak EGFP levels in plateau cultures were approximately 25–50% of those observed in identically induced exponential cultures. Ostensibly, the differences in expression from the heat shock promoter observed in exponential and plateau cultures were attributable to cell division diluting the vector within exponential cultures and the lower metabolic activity in serum-starved plateau cultures. For all experimental conditions, EGFP expression induced from the heat shock promoter was comparable with or higher than that from the constitutively active cytomegalovirus promoter over any 24-h period.

The experimental results demonstrated that EGFP expression from the heat shock promoter was controllable in both exponential and plateau phase cultures and support the plausibility of using controlled heat shock activation of this promoter as a means of regulating both the spatial and temporal expression of therapeutic DNA constructs within human tissues. The ability to localize and regulate expression from the heat shock promoter may prove particularly advantageous for many cancer applications, especially if the therapeutic products are highly toxic, e.g., proteotoxins or cytokines. However, the results of this study suggest that differential growth conditions within tumors could markedly affect the expression of recombinant DNA under control of both inducible and constitutive promoters. Consequently, inducing schemes may need to be spatially adjusted to obtain the desired therapeutic results in all tumor domains using heat-activated gene therapy.

INTRODUCTION

The heat shock promoter is one of several inducible promoters offering the potential for controlled expression of therapeutic genes delivered into diseased or normal tissues. A major advantage of the heat shock promoter would be the ability to use conformal heat shock to control and delimit its expression within the body even when recombinant DNA vectors are administered systemically. Microwave (1–3), ultrasound (4–6), or low frequency radiofrequency (7) devices designed for localized clinical hyperthermia could be used to deliver conformal heat shocks to confine heat shock promoter activation, whereas the magnitude and temporal duration of the heat shock would regulate that of induced DNA expression (8–10).

Human tissues can be heated relatively quickly to temperatures capable of activating the heat shock promoter, e.g., 40.5°C–43.0°C, and heat diffuses rapidly from tissues once the heat source is removed. Consequently, the heat shock promoter can be activated over a wide temporal range, from minutes to hours. Promoters that are activated by systemically delivered compounds, e.g., the tetracycline promoter (11), are not necessarily subject to such strict temporal control unless the activating substance clears quickly from tissues and blood. However, systemically delivered activation does lack the spatial control of promoter induction available with the heat shock promoter, which can be activated in defined tissue volumes using conformal heating techniques.

Radiation-inducible promoters (12–14) could potentially provide tight spatial and temporal control of induced promoter activity because ionizing radiation doses can be delivered with great precision in defined tissue volumes. Unfortunately, radiation-inducible promoters exhibit substantial, constitutive expression, whereas that from the truncated heat shock promoter is virtually undetectable. In some circumstances, a baseline of constitutive expression from an inducible promoter may not be a problem. However, if the therapeutic gene is extremely cytotoxic, e.g., proteotoxins like diphtheria and Shiga toxins, even low levels of constitutive expression could prove very toxic to any normal tissues that inadvertently receives it.

Developing heat-activated gene therapy for treating human cancers is a logical approach to controlling gene therapy because hyperthermia is already being investigated as an adjuvant modality for cancer radiation therapy (15–18). Consequently, the devices used to produce local or conformal heating and the technical expertise to use them are already available to many radiation oncology departments for testing the feasibility of heat-activated gene therapy. If initial tests prove promising, heat-activated gene therapy could ultimately be administered concomitantly with hyperthermia and ionizing radiation treatments. The amount of administered, recombinant DNA would need to be adjusted so that desired levels of therapeutic gene product were maintained throughout the fractionated regimes typically used to deliver heat and ionizing radiation. Between-fraction boost heatings could be used to adjust gene expression locally, should that prove necessary. Collaborations with other departments could then lead to expanding heat-activated gene therapy to other medical applications.

Nonreplicating adenovirus vectors (19, 20) are particularly well suited for cancer therapy because the recombinant DNA they deliver into cells is transiently resident and does not recombine into the host cell genome. This is particularly important when the therapeutic gene product is very cytotoxic, which could lead to severe complications should the recombinant DNA become permanently incorporated into the genome of normal tissues.

One concern in using adenovirus vectors for gene therapy is their inactivation by the host’s immune system (21, 22), which has prompted attempts to circumvent this potential obstacle (23, 24). However, immune inactivation of adenovirus may not represent a problem within tumors (25). This may result, in part, from the abnor-
mal nature of tumor vascularization, which may restrict access of antibodies and immuno-active cells to tumors (26) and/or reduce stimulatory interactions between these cells and the tumor vascular endothelium (27–29). Consequently, immune inactivation of adenoviruses in normal tissues may actually help focus expression of adenovirus vectors in tumors. Adenovirus vectors delivered directly into the tumor would be capable of infecting cancerous cells with minimal inactivation by the immune response, whereas vectors escaping the tumor might be inactivated by a sensitized immune system prior to their infecting normal tissues. Vector inactivation outside the tumor would prove most advantageous when the therapeutic DNA produces a toxic product. For these and other reasons, adenovirus vectors were selected for delivering recombinant DNA into the cells used in this study.

Many stresses other than heat shock elicit expression of HSPs, including oxidative stress (30) and suboptimal growth conditions (31, 32). Because these two induction conditions can exist within tumors, it is plausible that they could result in uncontrolled expression from the heat shock promoter. Truncated forms of the heat shock promoter exhibit induced expression by a more limited range of stresses (33, 34). As illustrated herein, a commercially available, truncated, human heat shock promoter (StressGen) exhibited abundant and controllable gene expression in response to heat shock while being virtually silent at 37.0°C and unresponsive to the suboptimal growth conditions of plateau-phase cultures. Fever still represents a potential for uncontrolled expression from the truncated heat shock promoter. However, treatment with antibiotics and other fever-reducing measures could potentially be used until either the fever-inducing event passes or the therapeutic gene construct is no longer functional, e.g., as with transiently infecting adenovirus vectors.

MATERIALS AND METHODS

Cell Culture. Du-145 human prostate carcinoma cells were adapted to growth in 10% iron-supplemented calf serum (Hyclone) in DMEM/F12, supplemented with MEM nonessential amino acids (Life Technologies, Inc.), MEM vitamin solution (0.5× recommended concentration; Life Technologies, Inc.), and 1 mM L-glutamine. The adaptation process required 3 weeks, and the adapted cells were designated Dut-145 cells.

Dut-145 cells were grown as monolayers at 37.0°C in humidified incubators with 5% CO2 to maintain medium pH at 7.4. For experimentation, cells were seeded into 25-, 75-, or 150-cm2 culture flasks to obtain cultures at 80–85% of confluency 48 h later. As illustrated herein, a commercially available, truncated, human heat shock promoter (StressGen) exhibited abundant and controllable gene expression in response to heat shock while being virtually silent at 37.0°C and unresponsive to the suboptimal growth conditions of plateau-phase cultures. Fever still represents a potential for uncontrolled expression from the truncated heat shock promoter. However, treatment with antibiotics and other fever-reducing measures could potentially be used until either the fever-inducing event passes or the therapeutic gene construct is no longer functional, e.g., as with transiently infecting adenovirus vectors.

Adenovirus Vector Construction. The plasmids and 293 cells used to produce nonreplicating adenovirus vectors were obtained from Microbix, Inc. Adenoviral vectors expressing EGFP under control of the truncated heat shock promoter were produced by first cloning the truncated heat shock promoter (StressGen, Inc.) into the adenovirus shuttle plasmids pAE1spA1 and pAE1spB1 (19), followed by cloning the EGFP gene sequence and poly(A) region from plasmid pEGFP-1 (Clontech, Inc.) downstream of the heat shock promoter. Adenoviral vectors expressing EGFP under control of the CMV promoter were produced by cloning the EGFP gene (from pEGFP-1) into the multiple cloning site of plasmid pCI-Neo (Promega, Inc.), after which the entire pCI-Neo expression cassette was excised and cloned into the adenovirus shuttle plasmids.

Adenovirus shuttle plasmids containing the EGFP expression cassettes were cotransfected (calcium phosphate) with plasmid PJM-17 into 293 cells (19). Recombination of the expression cassettes from the shuttle plasmids into PJM-17 yielded viral DNA of packageable size that produced adenovirus capable of replicating in 293 cells. Transfected 293 cells were overlaid with agarose to permit isolation of individual virus plaques.

Virus Clone Selection, Amplification, and Freezing. Virus clones were selected as agarose plugs of individual plaques, and ~10% of the virions from each plaque were used to infect 293 cells in a 60-mm culture dish. The 293 cells were harvested when they were cytopathic, pelleted, suspended in 5 ml of DMEM/F12 containing 5% heat-inactivated horse serum and 10% glycerol, and then frozen at -70°C. The cells were later thawed, and virions were freed by three freeze-thaw cycles using, alternately, liquid nitrogen and a 37.0°C water bath. Cell lysates were clarified by centrifugation for 10 min at 14,000 × g and separation of the lysate from the pellet. The lysate was stored at -80°C as aliquots, one of which was later thawed to titers the virus preparation (19). Each virus clone was then tested and selected for use in experiments based upon their ability to infect Dut-145 cells and express EGFP under control of either the CMV or truncated heat shock promoters.

Selected clones were further amplified to produce sufficient viruses for experiments by infecting larger quantities of 293 cells (30–300 100-mm culture dishes) at an MOI of 0.3. Cells were harvested when ~80% of the culture exhibited cytopathic morphology, and freeze-thaw cell lysates were centrifuged on cesium chloride step gradients at 60,000 × g for 2 h at 20.0°C (35) to separate viruses from defective particles and empty capsids. Recovered virus bands were dialyzed overnight into PBS. Glycerol was then added to 10%, and aliquoted virus suspensions were frozen and stored at -80°C. Again, one aliquot was thawed and used to titer the virus preparation.

Adenovirus vectors with [3H]thymidine-labeled DNA were produced in an identical manner, except that the infected 293 cells were cultured in medium containing 9.8 μCi/ml [3H]thymidine (84 Ci/mmol).

Adenovirus Infection of Experimental Cells. Exponentially growing cells were trypsinized from monolayer cultures, resuspended in DMEM/F12 containing 5% heat-inactivated horse serum (1.5 × 106 cells/ml), pelleted in 15 ml of polystyrene centrifuge tubes (5 min at 1200 rpm), and resuspended in the same medium at 1 × 106 cells/750 μl. The determined amount of adenovirus was added, the centrifuge tube was capped tightly and made watertight with a paraffin film, and each tube was mounted horizontally into a rack submerged in a 37.0°C water bath. The cell suspensions were agitated gently for 2.0 h by attaching the rack to a wrist-action shaker. Afterward, the cells were diluted appropriately into growth medium and then seeded into 35-mm culture dishes such that the cells would be at ~80–85% of confluency when assayed. For assay times longer than 72 h after infection, cells were maintained in exponential growth by subculturing. Many different virus infection procedures were tested, and this suspension method produced the most efficient, reproducible, and uniform (cell-to-cell) infections.

Assaying EGFP Produced in Virus-infected Cells. The 35-mm dishes were rinsed twice with PBS and aspirated dry, and the cells were scraped into 500 μl of reporter lysis buffer (Promega, Inc.). The samples were collected in triplicate and then frozen at -20°C for later fluorometric quantitation of EGFP content. For most experiments, triplicate samples were also collected for analyses by Western blotting (36).

A standard curve was established by determining the fluorescence of measured quantities of recombinant EGFP (Clontech, Inc.) using a Perkin-Elmer LS-50B spectrofluorometer (excitation, 480 nm; emission, 510 nm). Frozen lysates of experimental cells were thawed, mixed, and then assayed with the spectrofluorometer to determine EGFP fluorescence/volume. Each sample was also subjected to the BCA assay (Pierce, Inc.) to determine its protein concentration, which was used to calculate the amount of EGFP/μg of total cellular protein. This value was then converted to pg of EGFP/cell using predetermined values of the average protein content/cell.

Quantitative Western Blots. Quantitative Western Blots were performed as described previously (36), and measured quantities of recombinant EGFP (Promega, Inc.) were included in each gel to establish a standard curve for determining the EGFP content of experimental samples. The absorbance of all bands on the Western blot chemiluminesgraphs (ECL system; Amersham-Phar- macia) were measured with a laser densitometer.

RESULTS

Controlled, Induced Expression from the Heat Shock Promoter. The EGFP gene was silent under control of the truncated heat shock promoter in nonheated Dut-145 cells that were infected at an MOI of 40 or less. This is illustrated visually by both fluorescence micrographs (Figs. 1A and 2A) and Western blots (Fig. 1B and 2B). These figures also demonstrate that the magnitude of the heat shock
(time at temperature) at 41.0°C, 42.0°C, or 43.0°C, and adenoviral MOI determined the magnitude of EGFP expression. Heat shocks at 40.5°C induced barely detectable EGFP expression, even after infections at MOIs between 20 and 40 (data not shown). Induction at temperatures higher than 43.0°C produced progressively lower EGFP expression, ostensibly because the harsher heat shocks markedly reduced transcription and translation and caused significant cell killing (10, 37).

Using MOIs ≥40 had several undesirable effects upon the infected cells. The first was that EGFP expression could be detected in 2–5% of control, nonheated cells. Although the number of control cells expressing EGFP increased with MOI, the experiment to experiment variation was great and did not permit establishing a mathematical relationship for uninduced EGFP expression as a function of MOI ≥40. When the MOI was 120 or greater, all of the cells exhibited some level of uninduced EGFP expression, ceased dividing, had twice the diameter (measured from cells spread on the substrate) of the control cells, and exhibited elevated HSP levels (HSC-70, HSP-70, and HSP-90; data not shown). The latter indicated that infection with the adenoviral vectors placed the cells in a state of stress, most likely caused by overexpressing viral proteins, the genes of which are also present within the adenoviral vectors. This phenomenon is different...
from the stress induced by infection with replication-competent adenovirus, which results when the cells enter a cytopathic state as the replicating virus within cells distends them toward the point of rupture.

Results similar to those reported for the Dut-145 cells were also observed in three other cell lines, i.e., PC3 human prostate carcinoma, A549 human lung carcinoma, and HeLa human cervical carcinoma (data not shown).

**Temporal Profile of Heat-induced EGFP Expression.** Prior studies have demonstrated that the magnitude and temporal duration of stress-induced HSP synthesis, under control of their endogenous heat shock promoters, is proportional to that of the inducing stress (8, 9). The data in Figs. 1 and 2 depict induced EGFP expression at only one point after adenovirus infection and subsequent heat shock induction. Establishing a quantitative relationship between induction stress and temporal expression of therapeutic, recombinant DNA would be essential for effective and safe application of heat-activated gene therapy. Such data would serve as a guide to selecting the heat shock required for producing desired, dosage profiles of therapeutic DNA expression from the adenovirus vectors.

Fig. 3 shows that maximal EGFP expression from the truncated heat shock promoter was observed when exponentially growing cells...
Interestingly, the duration over which shocks longer than 2 h resulted in a progressive decrease in peak; 40% lower when cells were infected at MOI 20. Using 42.0°C heat identical to that induced by 41.0°C in cells infected at MOI 10 but was 42.0°C resulted from a 2-h heat shock. This maximum was essentially induction by 42.0°C. The maximal peak EGFP expression induced at 42.0°C, with cells infected at MOI 10 exhibiting the lowest ratios after between 0.85 and 1.1 following 41.0°C and 0.33 and 0.6 following expression at 12 h to that at 24 h after heat shock. This ratio ranged after heat shocks at 42.0°C (Fig. 5), as illustrated by the ratio of EGFP EGFP expression levels. Shocking cells at earlier or later postinfection times yielded lower EGFP expression, with the ability to induce significant expression being lost 90–100 h after infection. The majority of this loss was attributed to cell division diluting the number of EGFP gene copies/cell because the adenoviral DNA does not replicate in the Dut-145 cells. This postulate of vector dilution by cell division was supported by the ability to protract the period over which maximal EGFP expression could be induced to >80 h after infection by treating cells with the cell cycle inhibitor aphidicolin (3.0 μM), which was administered 12 h after infection and maintained in the culture medium thereafter (data not shown).

Temporal profiles of heat shock-induced EGFP expression were obtained for cells heated at 41.0°C (Fig. 4) or 42.0°C (Fig. 5) after infection at an MOI of 10 or 20. On the basis of the data in Fig. 3, heat shocks were administered 24 h after adenovirus infection to induce maximal EGFP expression, and the EGFP content/cell was measured periodically thereafter.

Maximal EGFP expression occurred between 12 and 24 h after heat shock at 41.0°C, and this was not affected appreciably by increasing MOI. After identical heat shocks, the peak-induced EGFP levels for cells infected at MOI 20 were 2-fold or more greater than those infected at MOI 10, and the decline from peak expression was slower. A 4-h heat shock yielded the maximum in both peak and temporal duration of EGFP expression after exposure to 41.0°C. Longer heat shocks at 41.0°C, up to 8 h, produced an EGFP expression profile identical to that induced by a 4-h exposure (data not shown). Heat shocks longer than 8 h at 41.0°C produced proportionately lower EGFP expression levels.

Compared with induction at 41.0°C, EGFP expression was delayed after heat shocks at 42.0°C (Fig. 5), as illustrated by the ratio of EGFP expression at 12 h to that at 24 h after heat shock. This ratio ranged between 0.85 and 1.1 following 41.0°C and 0.33 and 0.6 following 42.0°C, with cells infected at MOI 10 exhibiting the lowest ratios after induction by 42.0°C. The maximal peak EGFP expression induced at 42.0°C resulted from a 2-h heat shock. This maximum was essentially identical to that induced by 41.0°C in cells infected at MOI 10 but was ~40% lower when cells were infected at MOI 20. Using 42.0°C heat shocks longer than 2 h resulted in a progressive decrease in peak EGFP expression (Fig. 5). Interestingly, the duration over which induced EGFP could be detected was relatively refractory to heating time or the use of 41.0°C or 42.0°C.

Spectrofluorimeter measurement of EGFP proved usable over a wider range of cellular EGFP levels than measurement by Western blots (data not shown), possibly because the linearity between EGFP levels and fluorescence was more expansive than that for film density for exposed protein bands on chemilumigraphs. In addition, the spectrofluorimeter measurements were easier to make (especially for numerous samples) and yielded greater reproducibility. Consequently, the spectrofluorimeter method of quantitating cellular EGFP was used throughout this study. Spot checks of the spectrofluorimetric data by quantitative Western blots showed good agreement between the two methods (data not shown).

Depending upon the nature of the recombinant DNA being used for therapy, it might be desirable to either re-induce or protract its expression under the heat shock promoter. These issues were addressed by delivering 2-h, 41.0°C heat shock fractions at 24-h intervals, starting 24 h after virus infection. Fig. 6 shows that this fractionated heat shock regime extended EGFP expression such that it was still ~75% of peak 120 h after the initial heat shock (Fig. 6). Fractionated heat shocks at 24-h intervals may not have been optimal for sustaining maximal EGFP expression; however, Fig. 6 shows clearly that fractionated heat shocks markedly protracted high level EGFP expression without requiring infection with additional virus vectors.

Expression from the CMV Promoter. To be effective, gene therapy vectors must produce sufficient recombinant product to treat the
diseased state successfully. Many investigators have used strong, constitutive promoters, e.g., the CMV promoter, to maximize transgene expression to achieve this goal. Experiments were performed using adenovirus vectors wherein EGFP was expressed under control of the CMV promoter to determine how induced expression from the truncated heat shock promoter compared with a strong constitutive promoter that was already being used clinically.

Fig. 7 presents the temporal profile of EGFP expression from the CMV promoter in exponentially growing Dut-145 cells infected with adenovirus vectors at MOIs of 10, 20, or 30. For each MOI, cellular EGFP increased to a peak value that occurred 58–65 h after virus infection; however, the peak for the MOI 10 infection is not obvious from this figure because EGFP expression was so low relative to that following the higher MOI infections. As with the heat shock promoter, the temporal expression peaks were asymmetric, with a distinct shoulder following the peak.

The peak EGFP levels achieved after the three different MOIs were clearly not proportional to MOI (Fig. 8). Hence, the nonproportional EGFP expression was attributable to nonlinear CMV promoter activity.

A direct comparison shows that maximal expression from the heat shock promoter, after a 41.0°C, 2-h heat shock (Fig. 4), was 8- and 1.3-fold greater than that from the CMV promoter for cells infected at, respectively, MOI 10 and MOI 20. Maximal expression from the heat shock promoter was still ~8-fold greater after a 42.0°C heat shock to cells infected at MOI 10. However, in cells infected at MOI 20, peak expression from the CMV promoter was 1.15-fold greater than from the heat shock promoter when expression from the latter was induced by 42.0°C.

This peak production comparison demonstrates that the truncated heat shock promoter was either more effective than or equally effective as the CMV promoter, depending upon the MOI and induction temperature used. In actuality, the heat shock promoter was more productive because it was not transcribing continuously after the inducing heat shock. Prior studies have shown that transcription from the heat shock promoter ceases 4–8 h after induction, depending upon the intensity of the inducing stress (8–10, 30). Consequently, if one compares the amount of EGFP produced from the CMV and heat shock promoters over any 6- or 12-h period, the heat shock promoter is always found to be more productive.
Expression from the Heat Shock and CMV Promoters in Plateau Phase Cells. Human tumors consist of cells exhibiting growth rates ranging from plateau phase to exponential. Furthermore, most cells in normal tissues grow more slowly than those in the exponential cultures used for the experiments presented thus far. Consequently, it was relevant to investigate expression from the heat shock and promoters in plateau phase cultures.

Maximum EGFP expression from the heat shock promoter was induced when plateau phase cells were heated 48 h after infection with the adenovirus vectors (Fig. 9A) rather than 24 h after infection, as with exponential cells (Fig. 3). The maximal EGFP level induced was approximately one-third of that expressed in exponential cells (compare maximum levels in Figs. 4B and 9), but it still occurred 12–24 h after the heat shock (Fig. 9B). The postinfection period over which near-maximal EGFP expression could be induced from the heat shock promoter was markedly protracted in plateau phase cultures (Fig. 9A). Almost 70% of the peak expression level could still be induced 96–120 h after virus infection, whereas <10% of maximal EGFP expression could be induced 96 h after infection of exponential cells (Fig. 3). Furthermore, EGFP levels decayed more slowly from the peak induced level after heat induction in plateau phase cells (Fig. 9B). Fig. 9 also demonstrates that the plateau phase did not constitute a stress that induced significant expression from the heat shock promoter without administering an inducing stress.

Fig. 10 clearly shows that EGFP expression under control of the CMV promoter was delayed in plateau phase cultures, reaching peak values 96–120 h after infection instead of 55–65 h after infection, as in exponential cultures (Fig. 7). Expression also never achieved the same peak levels observed in exponential cells. However, cellular EGFP remained at peak levels longer, showing very little decrease even >200 h after virus infection. It is notable that EGFP expression in plateau cells infected at MOI 10 was higher than in identically infected exponential cells (Fig. 10). Conversely, the peak EGFP levels achieved after infections at MOI 20 and MOI 30 were less than in exponential cultures, with the biggest decrease noted in the MOI 30-infected cells.

DISCUSSION

This study demonstrated that the magnitude and temporal duration of transgene expression induced from a truncated heat shock promoter can be controlled in both exponential and plateau phase cultures of human tumor cells infected with adenovirus expression vectors carrying the transgene construct. Expression from the heat shock promoter was virtually undetectable until a heat shock >40.0°C was delivered, even in cultures that were maintained in plateau phase for >2 weeks. Once the threshold temperature was exceeded, transgene product was expressed in a dose-responsive manner that was proportional to the temperature and duration of the inducing heat shock. Sapareto and Dewey (38) established a time-temperature relationship for hyperthermic cell killing, which, in simplistic terms, states that equivalent levels of cell killing can be attained at different temperatures by decreasing the heating time by a factor of two for each degree the temperature is raised. The dose response for heat shock-induced transgene expression did not follow this time-temperature relationship strictly; however, the conformity was sufficient for the relationship to be used to estimate the relative amount of heat shock-induced transgene expression for different time-temperature combination heat shocks (Figs. 1 and 2). Unfortunately, the estimates were good over a limited time-temperature range and quickly became inaccurate when the heat shock was severe enough to induce significant inhibition of cellular metabolism and/or cell killing.

The current paradigms for heat shock activation of the stress response and hyperthermic cell killing incorporate thermal denaturation of cellular proteins as a critical event. Denatured proteins putatively interact with HSPs bound to heat shock transcription factor monomers such that they are freed to trimerize into the active form of the transcription factor, whereas aggregation of heat-denatured pro-
of the target tissue. Conversely, once CMV expression vectors are delivered, transgene expression is unregulatable and committed to a given level of transgene product. Additional vectors could be administered if expression from the CMV vectors is too low to be effective, but options would be limited if expression becomes too high. If vectors containing the CMV promoter infect normal tissues, there is no way to suppress its expression. If the transgene product is toxic or otherwise affects normal tissue function, the consequences of such inadvertent, unwanted transgene expression in normal tissues could be dire (39).

Growth conditions of the cells markedly affected transgene expression from both the CMV and heat shock promoters. Relative to exponential cultures, a much longer postinfection time was required to achieve the maximum, cellular EGFP level from the CMV promoter in plateau phase cells, and the postinfection time at which maximal expression could be induced from the heat shock promoter was delayed from 24 h in exponential cultures to 48 h. Once expression commenced or was induced in plateau phase cells, the maximal level of EGFP attained by the CMV or heat shock promoter, respectively, was reduced significantly, but these maximum EGFP levels were sustained for a significantly longer period.

The observed attenuation of the maximal amount of transgene product generated per cell could prove problematic for gene therapy in cells growing in suboptimal conditions, e.g., within the central regions of tumors. The therapy would fail if the attenuated maximum fell below the threshold level of transgene product required to achieve clinical efficacy. If, however, the temporal integral of the transgene product determined treatment efficacy, attenuation of transgene expression might be offset to some extent by the protracted period over which the maximum level of gene product persists in plateau phase cells.

The protracted period over which maximal, cellular EGFP levels were sustained in plateau phase cultures was greater for the CMV promoter, ostensibly because it remained constitutively expressed while expression from the heat shock promoter decayed after the inducing heat shock. This difference between the two promoters could be significant in normal tissues, where cell proliferation is usually much less than in exponential cell cultures, yet nutrients for optimal metabolism are abundant. Consequently, it is likely that CMV-controlled expression in normal tissues would be at the high level observed in exponential cultures and for the more protracted period observed in plateau phase cultures. This could result in uncontrollable, adverse side effects should too much normal tissue become infected with therapeutic vectors using the CMV promoter. The lack of induced expression in plateau cultures bodes well for the heat shock promoter being silent in nonheated normal tissues, wherein cells generally proliferate more slowly than in culture. If more protracted expression from the heat shock promoter is desired within a target tissues whose cells are in plateau phase, this can be accomplished using multiple heat shocks to sustain transgene expression (Fig. 9A).

Experiments with radioactively labeled viruses demonstrated that adenovirus infection efficiency and the time required for virus uptake in plateau phase cells were similar to those in exponential cultures. Consequently, it was postulated that the time required to process the adsorbed viruses in plateau phase cells and/or for viral DNA to migrate to the nucleus were the rate-limiting steps that delayed CMV expression and the postinfection time at which maximum expression could be induced from the heat shock promoter. Reduced metabolism resulting from nutrient-poor medium and intercell competition were most likely responsible for the attenuated EGFP expression in the plateau phase cultures. The protracted time over which cells maintained maximal EGFP levels was attributed to reduced cell proliferation in plateau phase cultures, a conclusion that was supported by experiments with cell cycle inhibitors.

Spatial control of heat shock promoter expression was not investi-
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gated, but its potential use for conformal gene therapy can be envis- 
ioned, as long as temperatures do not exceed 40.0°C in tissues, where 
expression is not wanted. Strong conformal control of promoter in- 
duction would be particularly important when the therapeutic trans- 
gene is toxic, e.g., a proteotoxin. In this instance, serious local 
complications could arise if undesired expression occurred in normal 
tissues surrounding diseased tissues injected with adenoviral vectors, 
with the potential for more serious, global complications if vectors are 
administered systemically. The controlled inducibility of the heat 
shock promoter demonstrated in this report leaves open the potential 
for systemic delivery of a wide range of gene therapy vectors, even 
those expressing cytotoxic products. Vector expression would occur 
only in selected, heat shocked sites. A major caveat would be the 
danger of fever activating the heat shock promoter and resulting in 
wide-spread toxicity. The potential for such a disaster, and the possi-
bility of averting it by using high doses of systemically administered 
antibiotics to control fever, need to be tested in animal models.

The ability to target the delivery and/or expression of gene therapy 
vectors to diseased tissues would greatly reduce the risks of inadvert-
ent expression in normal tissues. Although some promising data 
with tissue-specific promoters have been reported, considerable work re-
 mains before they are used routinely. Furthermore, there are many 
tissues for which specific promoters are not available, and wherein 
 an inducible promoter such as the heat shock promoter remains a good 
option for conformal gene therapy.

The results of this study support the feasibility for developing 
conformal, heat-activated gene therapy and provide data that can be 
used to anticipate the response of the heat shock promoter in vivo 
experiments. Additional in vitro and in vivo experiments are required 
to address the many concerns and questions that must be answered 
before heat-activated gene therapy can be introduced into the clinic. 
Clinical devices for delivering the necessary, local hyperthermia in the 
clinic of the range of 40.0°C to 42.0°C exist, and there should be little difficulty in 
 adapting these instruments for this application. The more daunting 
tasks will involve designing effective therapeutic vectors, delivering 
effective amounts of the vectors throughout diseased tissues, and 
understanding how the biology and physiology of different tissue 
 and its therapeutic outcome.

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activation of heat shock protein expression in human breast cancer cells. Cancer 
promoter demonstrated in this report leaves open the potential for systemic delivery of a wide range of gene therapy vectors, even those expressing cytotoxic products. Vector expression would occur only in selected, heat shocked sites. A major caveat would be the danger of fever activating the heat shock promoter and resulting in widespread toxicity. The potential for such a disaster, and the possibility of averting it by using high doses of systemically administered antibiotics to control fever, need to be tested in animal models.

The ability to target the delivery and/or expression of gene therapy vectors to diseased tissues would greatly reduce the risks of inadvertent expression in normal tissues. Although some promising data with tissue-specific promoters have been reported, considerable work remains before they are used routinely. Furthermore, there are many tissues for which specific promoters are not available, and wherein an inducible promoter such as the heat shock promoter remains a good option for conformal gene therapy.

The results of this study support the feasibility for developing conformal, heat-activated gene therapy and provide data that can be used to anticipate the response of the heat shock promoter in vivo experiments. Additional in vitro and in vivo experiments are required to address the many concerns and questions that must be answered before heat-activated gene therapy can be introduced into the clinic. Clinical devices for delivering the necessary, local hyperthermia in the range of 40.0°C to 42.0°C exist, and there should be little difficulty in adapting these instruments for this application. The more daunting tasks will involve designing effective therapeutic vectors, delivering effective amounts of the vectors throughout diseased tissues, and understanding how the biology and physiology of different tissues and their therapeutic outcome.

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