Gene Ther Mol Biol Vol 5, 1-8, 2000

A novel expression vector induced by heat, -radiation and chemotherapy

Research Article

Farha H. Vasanawala[1], Tom Tsang[1], Abdul Fellah[2], Peter Yorgin[2] and David T. Harris[1]*

[1]Department of Microbiology & Immunology and [2]Department of Pediatrics, University of Arizona, Tucson, AZ. USA.

*__Correspondence__: David Harris, Ph.D, Dept. Microbiology and Immunology, Bldg # 90, Main Campus, University of Arizona, Tucson, AZ 85721 USA. Tel: (520) 621-5127; Fax: (520) 621-6703; E-mail: davidh@u.arizona.edu

Key words: expression vector, inducible promoter, heat, radiation, chemotherapy

Abbreviations: beta-galactosidase, (-gal); cytomegalovirus, (CMV); Enhanced Green Fluorescence Protein, (EGFP); heat shock protein 70B, (HSP 70); Interleukin-2, (IL-2)

Received: 19 April 2000; accepted: 29 April 2000; electronically published: February 2004

Summary

In many gene therapy applications and molecular biology manipulations it is desirable to be able to control the expression of the therapeutic gene. In this study a new expression vector, pHOT-MCS, was constructed using a 451 bp fragment from the human heat shock protein 70B (HSP 70) promoter. The vector has a large multiple cloning site and the neomycin selectable marker, making it more user-friendly. Using the human Interleukin-2 (IL-2) gene as a marker, it was demonstrated that heat can induce the pHOT-IL-2 plasmid to express 2 fold more IL-2 than levels obtained with the human cytomegalovirus (CMV) promoter. Using the Enhanced Green Fluorescence Protein (EGFP) gene as a reporter gene, a human breast carcinoma cell line (MCF-7) was transfected with pHOT-EGFP and stably transfected cells selected with neomycin. The stable transfectants were subjected to three different experimental conditions; heat treatment at 42°C for one hour, treatment with geldanamycin at 1μg/ml (an anti-leukemic drug) or 3000 rads of -radiation. EGFP expression was measured for up to 72 hours by flow cytometry. The non-treated cells expressed a basal level of EGFP that increased 387% above background at 24 hours after heat-shock. Cells treated with Geldanamycin had a 208% increase in EGFP intensity at 24 hours which was maintained up to 72 hours as compared to the non-treated cells. Exposure of cells to 3000 rads of -radiation had a 150% increase in EGFP expression at 48 hours post-treatment as compared to the non-treated cells. Induction of heat shock proteins by heat, radiation and geldanamycin was confirmed by Western blot analysis. This inducible gene expression system may be applicable to clinical use in synergy with other types of standard therapy (e.g, hyperthermia, radiation and chemotherapy).

I. Introduction

The major therapies currently used to treat cancer include radiotherapy, chemotherapy, hyperthermia and immunotherapy. Gene therapy is emerging as a new treatment for cancer, with encouraging clinical results. With the emerging view of combinatorial therapy as an approach to cancer treatment (Feyerabend et al, 1997; Otte, 1988), there is a need to integrate gene therapy with conventional therapies. Most gene therapy approaches have utilized constitutive expression of therapeutic genes (e.g. co-receptors such as B7.1 and B7.2, cytokines such as IL-2 and GM-CSF) by viral promoters (e.g, cytomegalovirus and Rous sarcoma virus). An alternative method is the use of expression vectors that can be induced to express therapeutic genes by one or more of the aforementioned conventional therapies. Thus, vectors with inducible gene expression could be advantageous in some gene therapy protocols.

The human HSP70B promoter is a well characterized promoter (Schiller et al, 1988) known to be induced by a family of heat shock factors that respond to diverse forms of physiological and environmental stress including high temperatures, heavy metals, oxidative stress and anti-inflammatory drugs (Morimoto et al, 1997). The HSP70B promoter contains heat shock regulatory sequences that

bind to the heat shock transcription factor, thus "turning on" any gene downstream of the promoter. The HSP70B promoter has previously been incorporated in heterologous systems to express foreign genes in an inducible manner (Dreano et al, 1986). These vectors are difficult to use due to their large size (>10kb), small multiple cloning sites and lack of a selectable marker, such as neomycin.

In the present study a new user-friendly expression vector was constructed using a fragment of the HSP70B promoter and was tested for inducible gene expression. This promoter was compared to the CMV promoter, which has been shown in several studies to be one of the strongest promoters available (Boshart et al, 1985). Results showed that when heat-induced, the heat shock promoter fragment was twice as strong as the CMV promoter. Our results also demonstrated that this expression vector was inducible by -radiation and the anti-leukemic drug, geldanamycin (a benzoquinoid ansamycin antibiotic and a potent inhibitor of a protein kinase (Uehora et al, 1986) known to induce heat shock proteins (Conde et al, 1997)). The enhanced green florescence protein (EGFP) was used as a reporter gene to detect induction of the expression vector by the different treatments.

II. Results

A. Construction of vectors

The HOT-MCS vector (**Figure 1**) was generated by replacing the cytomegalovirus (CMV) promoter of pcDNA3 (Invitrogen, San Diego, CA) by a BamHI- Hind III fragment of the human HSP70B promoter from the p173OR plasmid (StressGen, Victoria, BC). The full-length HSP70B promoter is 2.3 kilo base pairs (kbp) in size, while the BamHI-HindIII fragment of the promoter is 451 bp in size. This adaptation resulted in a smaller vector, which was advantageous for further cloning. The pHOT-MCS vector also has a neomycin selectable marker that can be useful for *in vitro* research work. The pHOT-MCS vector has a large multiple cloning site which facilitates the cloning of genes into the vector. Thus, this newly constructed pHOT-MCS vector has many advantages over the p173OR plasmid.

B. Characterization and comparison of the inducible promoter expression vector

The pHOT-MCS plasmid containing a beta-galactosidase reporter gene (-gal) was evaluated for its ability to be induced by heat shock treatment. SW480 cells were transfected with the plasmid by the lipofection technique as described in Materials and Methods. Twenty-four hours after transfection the cells were heated to 42°C for one hour. Twenty-four hours after heat shock treatment the cells were stained for -gal gene expression. The p173OR plasmid expressing -gal under the control of the full-length 2.3kb HSP promoter was used as a positive control. The analyses (**Table 1**) revealed that -gal gene expression was not induced at 37°C, whereas the pHOT- -gal plasmid transfected cells stained positive 24 hours after heat treatment. Results were comparable with both vectors. These results confirmed that the 451 bp BamHI /Hind III fragment of HSP70B promoter maintained it's ability to be induced by heat shock.

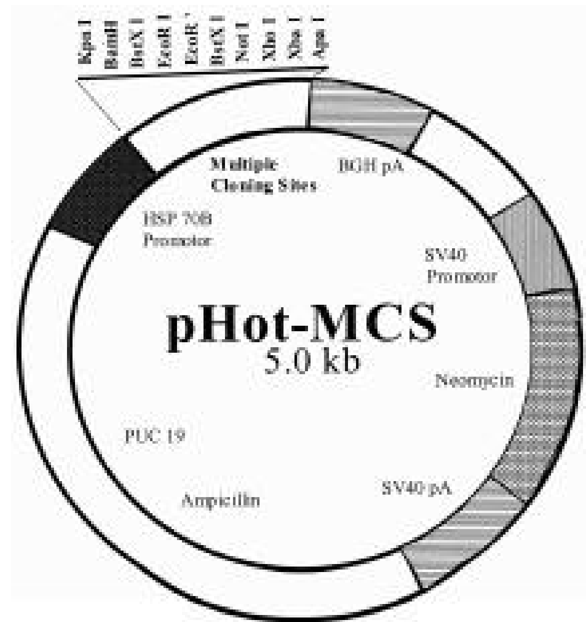

Figure 1. Map of the HOT-MCS expression plasmid. The plasmid was generated by replacing the CMV promoter of the pcDNA3 expression plasmid with a 400 bp BamH1/Hind III fragment of the human HSP70B promoter derived from the p173OR plasmid.

Table 1. Inducible gene expression by a fragment of the human HSP promoter. SW480 human colon carcinoma cells were transfected with either the HOT- -gal or the p173OR expression plasmid using the lipid DMRIE/DOPE as described in Materials and Methods. Transiently transfected cells were exposed for 1 hour to either 37°C or 42°C as described. Forty-eight hours after transfection the cells were harvested and stained for -gal using standard staining procedures (Invitrogen, San Diego, CA). Data are expressed as the percentage of cells staining positive for each treatment.

Percent -gal positive cells at:

PLASMID	37°C	42°C
p173OR	0%	27%
pHOT- -gal	0%	23%

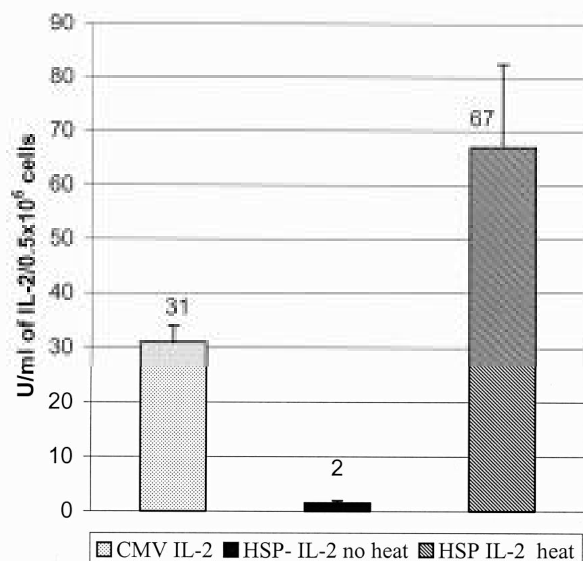

Figure 2. Promoter strength comparison by IL-2 production. MCF-7 cells were plated overnight at a density of 0.5x10^6 cells per well in a six well plate. The cells were transfected with the HOT-IL-2 plasmid using the lipid Novafector. The cells were heated to 42°C for 60 min and returned to 37°C. Twenty-four hours later the supernatants were harvested and analyzed by ELISA for IL-2 levels. Results are the mean of triplicate experiments.

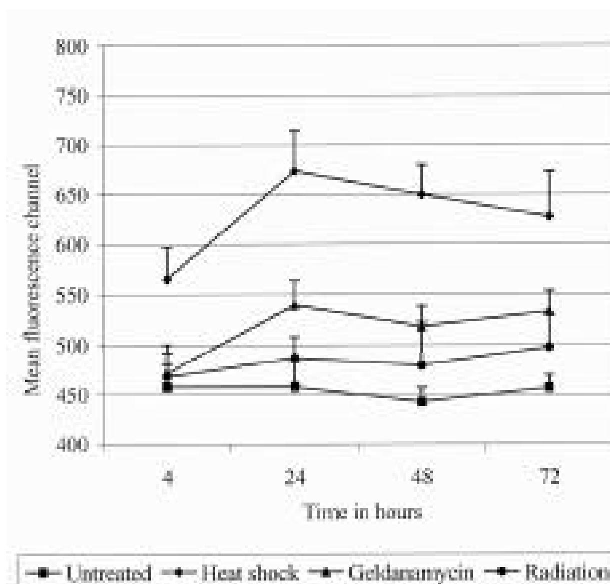

Figure 3. Increase in EGFP expression. Mean fluorescence channel (MFC) of EGFP expression following treatment with heat, radiation or chemotherapy. MCF-7 cells were treated as described in Materials and Methods, harvested at the indicated time points and analyzed by flow cytometry for EGFP intensity.

The promoter strength of the inducible expression vector was also tested against the CMV promoter. The IL-2 gene was cloned into the EcoRI site of the multiple cloning site of the pHOT-MCS vector. The HOT-IL-2 plasmid and the pcDNA3-IL-2 plasmid were transfected into MCF-7 cells with the lipid Novafector. Twenty-four hours after transfection the cells were heated and 24 hours after heat treatment supernatants were collected and assayed for IL-2. Results (**Figure 2**) showed that the pHOT-MCS vector promoter was twice as strong as the CMV promoter when induced by heat treatment (67 units v/s 31 units of IL-2).

C. Induction of the transgene by heat shock, -radiation and geldanamycin

Due to the difficulty in quantitating and comparing -gal assays and the expense of IL-2 assays, the EGFP reporter gene was cloned into the HOT-MCS plasmid yielding the HOT-EGFP plasmid. This plasmid can easily be detected by flow cytometry and was used for further experiments. MCF-7 cells were transfected with HOT-EGFP by the calcium phosphate method and stable transfectants were selected and maintained with the antibiotic G418. Stable, as compared to transient, transfections were chosen in order to eliminate differences in transfection efficiencies between experiments thereby allowing direct comparisons of increases in gene expression.

Stably transfected MCF-7 cells were plated overnight into 35mm, 6 well tissue culture plates at a density of 1x10^6 cells/plate. The next day adherent cells were treated with either heat (42°C, 1hour), -radiation (3000 rads, a previously determined optimal level) or geldanamycin (1µg/ml). Synergy of gene induction was also analyzed for by using -radiation and geldanamycin treatments together. Four, 24, 48, and 72 hours after treatment the cells were harvested and assayed for EGFP expression by flow cytometry. All transfected cells displayed a basal level of EGFP expression. Thus, results from the cell treatments are shown as the mean florescence channel (**Figure 3**) and as the percent increase in EGFP intensity (**Table 2**) over the basal level. Flow cytometric analyses indicated that heat shock treatment induced the highest level of gene expression. As early as 4 hours post-treatment there was a 243% increase in EGFP expression above the basal level. Maximal EGFP expression was observed at 24 hours after heat treatment (387% increase above background) after which it started to decline. Geldanamycin was also observed to induce the HSP promoter, with EGFP intensity increasing to 208% above background at 24 hours post-treatment. -radiation also induced the HSP promoter, although to a weaker extent than geldanamycin treatment. The highest level of gene expression was seen at 48 hours post-radiation treatment, with an increase of 150% over background levels. Finally, a combination of geldanamycin and radiation treatments together was not observed to increase gene expression (i.e.

act synergistically) above that seen with either treatment alone (data not shown). These results were significant in that it demonstrated that the HOT-EGFP vector could be induced not only by heat shock, but also by -radiation and chemotherapy.

D. Analysis of heat shock proteins by western blots

As the time course for induction of EGFP expression by both geldanamycin and -radiation treatments were different from that of heat-shock treatment, Western blots were performed on the treated cells at these time points to quantitate HSP72/73 production. As each of the treatments theoretically induced EGFP expression via the HSP promoter, the treatments should have induced an increase in cellular heat shock proteins. Western blot analyses (**Figure 4**) for HSP72/73 expression indicated that all three treatments (heat, radiation and geldanamycin) induced the HSP 72/73 proteins. The highest level of HSP 72/73 production was induced by heat at 4 hours post-treatment. However, at 24 hours post-treatment and thereafter, treatment with geldanamycin induced higher levels of HSP72/73 expression. -radiation also induced HSP72/73 protein expression at 4 hours post-treatment, but the levels were not significant at the later timepoints.

III. Discussion

We have constructed a novel, user-friendly inducible expression vector, that possesses a large multiple cloning site and the neomycin gene as a selectable marker. This vector has the useful property of being inducible by heat, chemotherapy, and radiation. Using pcDNA3 as the backbone plasmid, pHOT-MCS was derived by replacing the CMV promoter with a heat inducible promoter, the human HSP70B promoter.

As the size of a plasmid may affect transfection efficiency, such that smaller plasmids transfect with a higher efficiency than larger ones, size was an important factor in our plasmid design. Thus, a 451 bp fragment of the human HSP70B promoter was used rather than the entire 2.3kbp promoter. From previously published work (Schiller et al, 1988), the 451 bp fragment was expected to be as heat inducible as the parental promoter since it contains the heat shock element (HSE) sequences and the TATA box.

Results using -gal as the reporter gene indicated that the 451bp fragment of the human HSP70B promoter was indeed sufficient for heat inducible gene expression. Currently, the most commonly used promoter is the CMV promoter and it was imperative to compare the pHOT-MCS plasmid with this promoter. The results showed that the 451 bp fragment of the human HSP70B promoter upon treatment with heat was twice as strong as the CMV promoter. Thus, promoter strength was not compromised in the construction of the HOT-MCS plasmid.

In further experiments, EGFP was used as the reporter gene rather than -gal due to the ease of assaying EGFP gene expression by flow cytometry. Thus, the EGFP gene was cloned into the HOT-MCS plasmid, yielding the HOT-EGFP plasmid. The reporter gene was easily detected by flow cytometry (**Figure 3**). MCF-7 cells stably transfected with HOT-EGFP were used instead of transient transfections to eliminate differences in transfection efficiencies between experiments. Stably transfected cells allowed for direct comparisons in increased gene expression by the different experimental treatments. Since stable transfectants were used, EGFP gene expression was also observed in non-treated (but transfected) cells. This background expression was considered to be the background level and increases in gene expression were calculated as the fold-increase over the basal level.

It was a novel finding that the HSP70B promoter fragment was not only inducible by heat but also by -radiation and geldanamycin. These treatments represent radiation therapy and chemotherapy, and thus present an opportunity to combine gene therapy with existing cancer therapies such as hyperthermia therapy, chemotherapy and radiation therapy. Although, the fluorescence intensities with geldanamycin and -radiation weren't equivalent to that observed with heat shock, the levels were still significantly increased above basal levels.

	4 hours	24 hours	48 hours	72 hours
Heat	243	387	376	327
Geldanamycin	120	208	201	201
Radiation	113	138	150	154

Table 2. Percent Increase in EGFP Intensity. Mean Fluorescence Channel units from Figure 3 were converted into percent intensity increases above background. An increase in 75 units of MFC = a doubling in intensity. The above formula was used to calculate the percent increase in EGFP intensity over background, which was considered to be 100%.

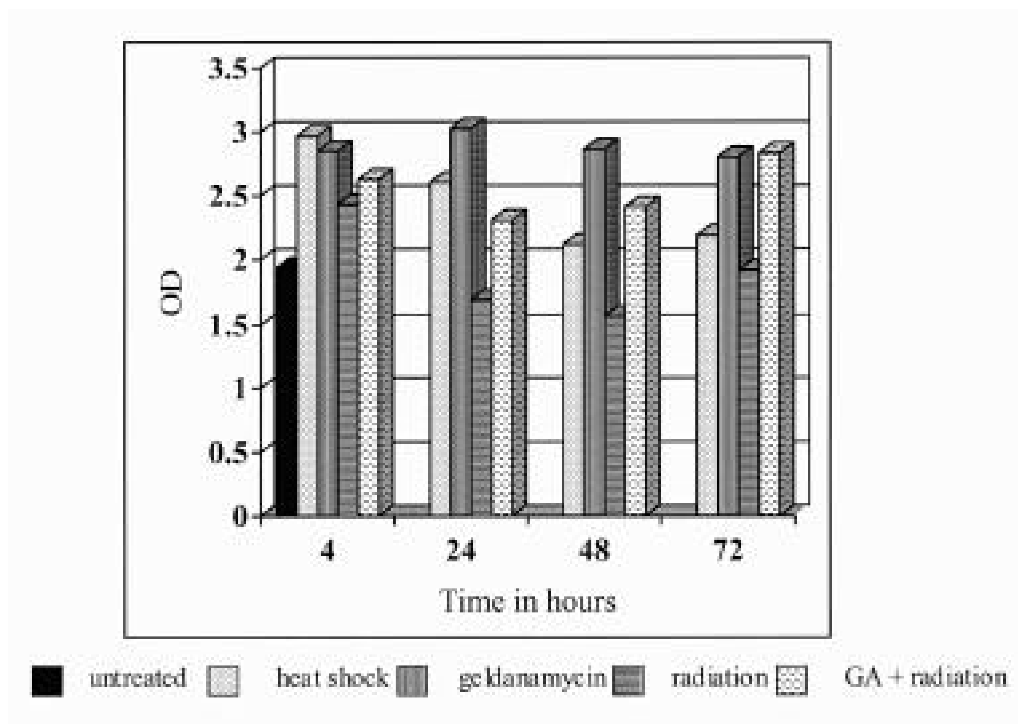

Figure 4. HSP72/72 protein expression after treatment with either heat, radiation or chemotherapy. MCF-7 cells were treated as described in Figure 2 and heat shock protein expression quantitated by western blotting and densitometry at the indicated time points. Data are presented optical density for each measurement.

There was a difference in the induction of gene expression between the three treatments. Heat shock induced gene expression rapidly by 4 hours, while maximal gene induction was seen at 24 hours with geldanamycin and -radiation treatments. Thus, different mechanisms of heat induction occurred with geldanamycin and -radiation. As these various treatments turned on the reporter gene, each of the treatments must have induced heat shock proteins in the cell since the heat shock promoter was activated. Thus, western blots detecting heat shock proteins were performed to possibly elaborate the mechanisms of gene induction. The western blot experiments indicated that heat shock increased HSP72/73 production at 4 hours and a similar effect was also seen with geldanamycin treatment. This increase in HSP72/73 levels was however, not reflected in an increase in EGFP intensity with geldanamycin treatment. Thus, it can be concluded that different mechanisms were affecting gene expression, which needs to be further characterized.

In conclusion, we have constructed a vector that opens up the possibility of a combinatorial type of therapy using gene therapy and chemotherapy, hyperthermia or radiation. Synergistic effects between these therapies may be more beneficial than any one therapy alone. Further, it may be possible to utilize lower doses of chemotherapy or radiation in combination with the above gene therapy vector expressing a biologically active gene (e.g, HSV-tk or cytokines) to achieve less toxic clinical results.

IV. Materials and Methods

A. Vector construction

Three reporter constructs were made from pHOT-MCS (see results for further details). One construct contained beta-galactosidase (-gal) as the reporter gene (from the pCMVB plasmid, Clontech, Palo Alto, CA) cloned into the Not I site of pHOT-MCS.

For the IL-2 construct, the human IL-2 gene (a gift from Dr. Evan Hersh, University of Arizona) was first adapted for EcoR1 site. Briefly, a 0.5kb BamHI-PstI DNA fragment containing the IL-2 gene was inserted into the Sac-KiSS- (Tsang et al, 1996) following a complete digestion with BamHI and a partial digestion with PstI to create the plasmid pSac-KiSS-IL-2. The IL-2 gene was then excised from the pSac-KiSS-IL-2 as an EcoRI fragment and inserted into the EcoRI site of pHOT-MCS to generate pHOT-IL-2. The third reporter construct contained EGFP (Clontech, Palo Alto, CA) as a reporter gene, which was inserted into the Kpn I – Not I multiple cloning site of pHOT-MCS.

B. Cell lines and transfections

MCF-7, a human breast carcinoma cell line, was transfected with the pHOT-EGFP vector by standard calcium phosphate methodologies (Sambrook et al, 1989). Stable transfectants were obtained by selection with G418 (400 µg/ml) and were maintained in complete RPMI medium, (GIBCO, Gaithersburg, MD) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenaxa, KS). The cells were maintained at 37°C in 5% CO_2.

For IL-2 studies, MCF-7 cells were seeded in 35mm plates (Falcon, Franklin Lakes, NJ) at $0.5x10^6$ cells/plate. Cells were

transfected with the plasmids using the lipid Novafector (VennNova, Pompano Beach, FL) at a ratio of 1µg DNA to 4 µl of lipid for 6 hours. SW480, a human colon carcinoma cell line, was transfected using DMRIE-DOPE (Vical Inc, San Diego, CA) (Parker et al, 1996).

For -gal studies, SW480 cells were plated out at $4x10^5$ cells/well in a six well tissue culture plate (Falcon, Franklin Lakes, NJ). A lipid to DNA ratio of 4:1 was used for transfections. Transfections were performed in reduced serum media OPTI-MEM (GIBCO, Gaithersburg, MD). Four hours after transfections, 0.5 ml of 30% FBS in OPTI-MEM was added to each well. The next day an additional 1ml of 10% FBS in OPTI-MEM was added. Forty-eight hours after transfection the cells were trypsinized and stained for -gal using the Invitrogen -gal staining kit (Invitrogen, Carlsbad, CA).

C. Cell treatments

Transfected cells were plated overnight in a $35mm^2$ tissue culture dish (Falcon, Franklin Lakes, NJ) at a density of $1x10^6$ cells in 5ml of RPMI medium. The following day the cells were treated as follows. Heat shock treatment was performed by sealing the plate with parafilm and immersing it in a 42°C water bath for 60 min. Geldanamycin (Sigma, St. Louis, MO) was added to the cell cultures at a concentration of 1µg/ml. -radiation treatment was performed using a ^{60}Co - irradiation unit. The cells were exposed to a total of 3000 rads (225 rads/min) of radiation in a single dose. All treated cells were trypsinized and assayed by flow cytometry for EGFP expression at 4, 24, 48 and 72 hours post-treatment.

D. IL-2 assay

Cell supernatants were harvested 24 hours after heat treatments. The supernatants were assayed by ELISA for IL-2 levels using MEDGENIX IL-2 EASIA Kit (Biosource Europa, Belgium).

E. Flow cytometry

Flow cytometric analysis was performed using a FACStar ^{PLUS} flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Data was acquired utilizing a COHERENT (Palo Alto, CA) 90-5 5W argon ion water-cooled laser tuned to 488 nm at 100mW power for excitation. Emitted fluorescence was collected with a standard 530/30 band pass filter. A minimum of 10,000 events were collected in a 'live' gate. Data was acquired and analyzed on an HP340 computer with Lysys II (Becton Dickinson Immunocytometry Systems Mountain View, CA) software. Data was collected as mean florescence channel of EGFP. An increase in 75 units of mean florescence channel indicates a doubling in fluorescence intensity (as per personal communications from Becton Dickinson, according to the formula:

$$\frac{\text{channel value}}{\text{channel per decade}} = 10^x$$

where x= log channel

Data is represented as the percent increase in EGFP intensity over background intensity.

F. Western blots

Western immunoblots were performed to estimate production of heat shock proteins.

Cells to be assayed were washed with phosphate buffered saline and resuspended in cell lysis buffer. Total protein was estimated by the BCA protein assay (Pierce, Rockford, IL). Protein samples (30mg each) were fractionated for Western blot analysis by separation on denaturing SDS-PAGE and transferred onto nitrocellulose filters. Filters were blocked by soaking the membrane in buffer containing 3% milk in TTBS (Tris-buffered saline containing 1% Tween-20) to minimize non-specific binding. After three washes in TTBS the membranes were incubated with anti-HSP 72/73 antibody (StressGen, Victoria, BC). Goat anti-mouse IgG-horse radish peroxidase (Pierce, Rockford, IL) was used as a secondary/developing antibody. Both incubations were performed at room temperature for 1 hour. The membrane was washed three times with TTBS and incubated in substrate reagent containing peroxide for 5 min. Heat shock proteins were detected by exposure to ELC hyperfilm and developed for autoradiogram. Heat shock protein expression was quantitated by scanning densitometry.

Acknowledgements

The authors would like to thank Dr. Ashok Gupta for his technical assistance. The authors would also like to thank Barb Carolus for all her help with flow cytometry.

For plasmid requests please contact Dr. David Harris at davidh@u.arizona.edu or write to Dr. David T. Harris. Dept. of Microbiology and Immunology, Building # 90, University of Arizona, Tucson, AZ 85721.

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