Use of the Stress-inducible grp78/BiP Promoter in Targeting High Level Gene Expression in Fibrosarcoma In Vivo

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Abstract

Current advances in human gene therapy open up new frontiers for molecular therapies of cancer. However, one major limitation in cancer gene therapy is the lack of a general tumor-specific promoter which allows stringent and high level expression of the therapeutic reagent in malignantly transformed but not normal tissues. Hallmark features of solid tumors such as glucose deprivation, chronic anoxia, and acidic pH induce glucose-regulated proteins, in particular, GRP78/BiP, a 78,000 endoplasmic reticulum-localized protein with chaperone and calcium-binding properties. We report here that a truncated rat grp78 promoter with most of the distal basal elements removed can be utilized as a potent internal promoter in a retroviral vector to drive high level expression of a reporter gene in a murine fibrosarcoma model system. The stress-inducible grp78 promoter offers a novel approach for gene delivery systems targeting transcription in tumorigenic cells.

Introduction

Glucose deprivation, chronic anoxia, and acidic pH known to persist in solid tumors with poor vascularization induce GRPs (1-3). For example, GRP78, also known as BiP, is induced about 10-fold under these stress conditions, primarily through transcriptional control (1). As molecular chaperones and calcium-binding proteins localized in the endoplasmic reticulum, elevated levels of GRPs protect cells against physiological adverse conditions (4, 5). Increased GRP78 expression is detected in virus-, chemical-, and radiation-transformed cells (6). In B/C10ME tumor cells, resistance to cell-mediated cytotoxicity is mediated by GRP induction (7). During growth of a murine tumor from radiation-induced fibrosarcoma, GRPs are increased, correlating with size of the tumor (8). Thus, GRPs are major cellular proteins specifically induced in transformed cells and in tumor environments to confer a protective role in cell survival.

For successful administration of gene therapy in cancer, the ability to achieve high level gene expression in a tumor-specific manner will be extremely useful since it could alleviate the need for precise targeting of the gene delivery system (9). Current efforts in designing promoters in gene delivery systems have focused mainly on using strong viral and cellular promoters to drive the expression of the foreign gene. Commonly used viral promoters include Moloney murine leukemia virus and HaMSV enhancer/promoter, both located in the retrovirus LTR (10, 11). A potent viral promoter derived from cytomegalovirus is used widely as an internal promoter to enhance gene expression in retroviral vectors (12). Cellular promoters from the phosphoglycerate kinase, β-actin, and histone genes (13) have also been used as internal promoters with varying degrees of success in a number of cell lines and tissues. While these viral and cellular promoters are effective in driving high level expression, nonetheless, their effect is constitutive and cannot offer tissue- or tumor-specific selectivity.

Recently, tissue-specific cellular promoters have been identified which allowed targeted gene expression in vivo. For example, a 2.5-kilobase fragment of the tyrosinase promoter stringently restricts the expression of the reporter gene in melanomas, although normal melanocytes infected with the retroviral vector will also express the reporter gene (14). In a transgenic mouse model, a 7.6-kilobase fragment of the 5'-flanking sequence DNA of the mouse fetal α-fetoprotein gene, which can be abnormally reactivated in hepatocellular carcinoma, has been exploited to direct expression of a foreign gene in liver cancers (15). Unique properties of the stress-inducible grp78 promoter suggest that it may offer special advantages for high level expression in malignantly transformed cells and a variety of solid tumors. grp78 is a single copy gene in mammalian cells, and its promoter, when fused to a reporter gene, is highly inducible by glucose-deprivation in vitro (16). While this gene is expressed at a low basal level in most tissues, the promoter elements controlling basal level expression are located upstream with the stress-response elements residing within 155 base pairs proximal to the TATA element (17). We report here that a truncated grp78 promoter with most of the distal basal elements removed can be utilized as a potent internal promoter in a retroviral gene delivery system to drive high level expression of a marker gene in a murine fibrosarcoma B/C10ME system.

Materials and Methods

Retroviral Construction. To construct pHaMAGRPNeo, PCR amplification was used to obtain a fragment of the grp78 promoter spanning nucleotides -599 to +33 (18), with a MluI site at its 5' end and a SalI site at its 3' end. The PCR fragment was cloned into the MluI-SalI sites of pHAma, which is described elsewhere (19) and which contains the MDRI gene as a selectable marker. A cDNA encoding the bacterial neoA gene flanked by SalI and XhoI sites was then inserted into the SalI site downstream of the grp78 promoter. For pHaMASV.neo, a MluI-SalI fragment carrying the SV40 early promoter (20) was removed from vector pSK1.MDR (21) and cloned into the same sites of pHaMa. The neo cDNA was inserted as above.

Retroviral Production and Titering on NIH3T3 Cells. NIH3T3 cells were maintained in MEM containing 10% bovine serum ( Irvine Scientific), 5 mm glutamine, 50 units of penicillin/ml, and 50 µg of streptomycin/ml. GP+EG6 and GP+envAm12 cells (kindly provided by A. Bank) were maintained in the same medium but with 10% fetal bovine serum. Plasmids pHaMAGRPNeo and pHaMASV.neo were transfected into GP+E86 ecotropic packaging cells (22) by the CaPO4-DNA coprecipitation method. Cells were selected in the presence of 20 ng/ml colchicinc (Sigma Chemical Co., St. Louis, MO) as described previously (21), and drug-resistant cells were pooled and grown to 80% confluence. Fresh medium lacking colchicine was added to
the cells, and viruses were collected after 20–24 h. Ecotropic viruses were used to transduce the GP+envAml2 amphotropic packaging cell line (23), transduced cells were selected in 20 ng/ml colchicine, and viruses were collected as above. Amphotropic virus supernatants were titered on NIH3T3 cells and determined to be 3.6 × 10^8 colony-forming units/ml for pHaMAGR neo and 5.6 × 10^8 colony-forming units/ml for pHaMASV.neo.

**Retroviral Transduction.** B/C10ME cells were grown in high glucose DMEM containing 4.5 mg/ml glucose supplemented with 10% FCS and 2 mM glutamine. Cells (5 × 10^6) were plated on 60-mm dishes. On day 1, the medium was changed with the addition of 8 μg/ml polybrene. The cells were infected with the virus at a multiplicity of infection of 1 for 48 h. On day 3, the cells were trypsinized and replated at 1:4 in growth media containing 60 ng/ml colchicine for the selection of MDR1 gene expression. On day 12, the surviving cells were pooled and expanded.

**RNA Isolation and Northern Blot Analysis.** Transduced or nontransduced B/C10ME cells grown in culture were injected s.c. into a BALB/c mouse and allowed to form tumors. The tumor growth was monitored by caliper measurements (Fig. 2A). In contrast, the level of neo mRNA driven by the SV40 promoter indicated that the level of neo mRNA driven by the internal grp78 promoter was low and not evident that the transduced cells were being stressed. Our results suggested that the promoters were active in the transduced cells.

**In Vivo Hybridization.** Sections were fixed in 4% paraformaldehyde (in PBS, pH 7) for 30 min at room temperature. Sections were then sequentially rinsed 3 times in PBS for 10 min. Afterward, slides were rinsed in RNAase-free water for 1 min. Next, sections were placed in 0.1 M triethanolamine for 1 min (pH 8). To 200 ml of 0.1 M triethanolamine, 0.5 ml of acetic anhydride was added and slides were rinsed in this solution for 10 min.Slides were then rinsed in RNAase-free water for 1 min, dehydrated with a series of ethanol concentrations (30, 50, 70, 85, 95, and 100%), and air dried. The in vitro-transcribed probes were generated as follows: a 3-kilobase grp78 hamster cDNA EcoRV/Sall fragment was cloned into the corresponding sites of the PBS vector (pBS plasmid adjacent to a T7 and T3 promoter). Prior to the in vitro transcription reaction, the grp subclone was digested with PstII to generate a 0.95-kilobase fragment which was transcribed as a probe. The grp antisense and sense probes were generated by using T7 and T3 polymerases, respectively, in the presence of [35S]UTP to yield the RNA probes. The grp antisense and sense probes were 950 bases pairs long. To generate the neo probe, pNeo was digested with PstI to generate a 0.92-kilobase fragment of the neomycin resistance gene-coding sequence. This 0.92-kilobase fragment was subcloned into the PstI site of PBS plasmid adjacent to a T7 and T3 promoter. T7 and T3 polymerases were used to yield the antisense and sense RNA probes, respectively. All the probes had a specific activity of about 1.0 × 10^8 cpm/μg of RNA. Two to three ng of RNA probe were used per slide for hybridization. The probes and slides were incubated at 50°C for 3 h in a moist chamber in a hybridization solution containing 50% formamide, 4X SSC, 5 X Denhardt’s, 1% SDS, 10% dextran sulfate, and 250 μg/ml tRNA. Slides were then soaked in 20 mm DTT/4X SSC for 10 min and placed in RNAase digestion buffer for 30 min at 37°C (5 mM NaCl-1 mM Tris, pH 8.0-5.0 EDTA-10 mg/ml RNAase A). The slides were then washed overnight in 2× SSC/0.2 m β-mercaptoethanol. The following day, slides were washed for 1 h in 0.1× SSC at 60°C. For high stringency wash, the slides were further incubated at 70°C for 1 h in 50% formamide, 0.5 m NaCl, 5% sodium phosphate, 0.2 m β-mercaptoethanol, and 1% SDS. All slides were dehydrated with a series of ethanol concentrations (30, 70, 95, and 100%), air dried, and exposed to film.

**Results and Discussion.** Retroviral vectors enabling us to compare directly grp78 as an internal promoter to the well-characterized SV40 viral promoter were prepared. As shown in Fig. 1, this set of retroviral vectors contained the human multidrug resistance gene MDR1 gene driven by the HaMSV LTR. MDR1 is a dominant, selectable, and amplifiable marker that allows selection of the transduced cells by colchicine (21). The neomycin resistance gene (neo) was used as a reporter gene driven by either the grp78 or the SV40 promoter. In the pHaMAGR.neo retroviral vector, the rat grp78 promoter fragment contained 632 base pairs upstream of the transcription initiation site. While it contained some residual basal elements, the stress-inducible regulatory elements located in two critical regions spanning -135 to -135 and -95 to -85 were fully retained (26, 27). The pHaMASV.neo vector was identical to the GRP vector except that it contained 340 base pairs of the immediate early SV40 promoter.

The B/C10ME cells were transduced with the retroviral vectors carrying either the grp78 or the SV40 promoter driving neo. These cells were chosen as recipients since they could form fibrosarcomas when injected s.c. into BALB/c mice. Further, the induction of GRP78 by various stress conditions is well documented in these cells (7). The transduced cells were selected by their MDR1-mediated resistance to colchicine, the expression of which was driven by the HaMSV LTR.

The two populations of transduced cells expressed equal levels of colchicine resistance. Further, this provided an unbiased selection since the level of colchicine resistance was not determined by the relative strengths of the internal grp78 or SV40 promoters being tested in these experiments. The transduction efficiency was high as estimated by the high fraction (~80%) of transduced cells which survived the colchicine selection. The resistant cells were pooled and expanded, and integration of the provirus was confirmed by PCR (data not shown). The two populations of transduced cells showed equivalent copy number of the integrated neo gene.

To measure the relative levels of neo expressed from the grp78 and SV40 promoter, total cytoplasmic RNA was extracted from B/C10ME cells and from the same cells transduced with vHaMAGR.neo or vHaMASV.neo virus. Prior to RNA extraction, the subconfluent cells were cultured for 30 h either in normal medium containing 4.5 mg/ml glucose or in glucose-free medium. neo and endogenous grp78 mRNA were measured by Northern analysis (Fig. 2A). Equal amounts of RNA were loaded on each lane, and based on the strong induction of the endogenous grp78 mRNA in the glucose starved samples, it was evident that the transduced cells were being stressed. Our results indicated that the level of neo mRNA driven by the internal grp78 promoter was induced 8-fold under glucose starvation conditions (Fig. 2B). In contrast, the level of neo mRNA driven by the SV40 promoter remained at the same low level under both culture conditions. The level of the longer neo-containing transcript driven by the HaMSV LTR in both the grp78 and the SV40 constructs was also low and not...
Fig. 2. RNA blot analysis of neo and grp78 mRNA levels in B/C10ME cells. A, B/C10ME cells were stably transduced with vHaMAGR neo (GRP.neo) or vHaMASV.neo (SV.neo). The cells were grown in either normal medium (+) or glucose-starved (GS) for 30 h prior to total cytoplasmic RNA extraction. Each lane contained 15 μg of RNA. The RNA blots were hybridized with either neo or grp78 probes. The autoradiograms are shown. The positions of the transcript driven by the viral LTR [neo (LTR)] and the internal promoter [neo (int.)] are indicated. Both transcripts contain neo sequences, although only the internal one is used for translation of the neo gene. Shown below are the endogenous grp78 mRNA levels in the corresponding RNA samples. B, quantitation of the neo (int.) mRNA levels in the B/C10ME-transduced cells (GRP.neo and SV.neo). The neo (int.) band intensities in A were scanned by laser densitometry. The level of neo (int.) in the GRP.neo cells grown under normal culture conditions (+) was set as 1. This value was used for the normalization of the other neo (int.) levels in the transduced cells grown either in glucose-containing (+) or glucose-free media under glucose starvation conditions (GS).

Fig. 3. In situ hybridizations of tumor sections. A, Tumors derived from B/C10ME cells stably transduced with vHaMASV.neo (Sections 1 and 2) or vHaMAGR neo (Sections 3 through 5) after s.c. injection in BALB/c mice were sectioned and hybridized to [35S]UTP-labeled probes to detect the endogenous grp78 mRNA (7K) level or background (bkg) hybridization as indicated on top. Sections 1, 2, and 3 were subjected to a high stringency wash. B, sections of tumors derived from vHaMAGR.neo-transduced cells were hybridized with either [35S]UTP-labeled neo (grp78/neo) probe or sense probe to detect background (bkg) as indicated on top. Two sample slides are shown. C, sections of tumors derived from vHaMASV.neo-transduced cells were hybridized with the probes as indicated on top. Two sample slides are shown.

Inducible by glucose deprivation (Fig. 2A). Quantitation of the neo mRNA driven by the internal promoters showed that the grp78 promoter had a 2-fold lower basal activity than the SV40 promoter (Fig. 2B). The low basal activity of the truncated grp78 promoter could be attributed to the deletion of much of the upstream enhancing sequence from the native promoter (18). Low basal level under normal growth conditions is important because an optimal promoter for tumor-specific expression would require that the promoter be suppressed in normal cells. Thus, the truncated grp78 promoter exhibited the desirable properties that (a) its basal level was lower than that of the HaMSV LTR and the SV40 promoter, and (b) while both HaMSV and SV40 promoters were nonresponsive to glucose-stressed conditions, the internal grp78 promoter was able to confer high inducibility to the reporter gene.

To examine whether the enhanced neo expression in glucose-starved cells in vitro could be reproduced in a tumor microenvironment in vivo where glucose supply might be limiting, B/C10ME cells stably transduced with vHaMAGR.neo and vHaMASV.neo were injected s.c. into BALB/c mice. After 3 weeks, progressively growing tumors (size, ~20 mm) were harvested and sectioned. In situ hybridization...
ized were performed with [35S]UTP-labeled “antisense” RNA probes able to detect endogenous grp78 and neo mRNA. To monitor for background hybridizations, equivalent sections were hybridized with the corresponding “sense” probes labeled to equal specific activities. Previously, with the use of RNA blots, it has been shown that grp78 mRNA levels are elevated in radiation-induced fibrosarcoma tumors, correlating with the size of the tumors (8). Here, using in situ hybridization, we observed strong hybridization of the grp78 probe to the tumor sections, in contrast to the uniform low background obtained with the control probe (Fig. 3A, compare sections 4 and 5). The level of grp78 was much lower in the muscle sections surrounding the tumor (data not shown). Upon a higher stringency wash, we detected pockets of higher intensity grp78 mRNA levels in localized, central regions of the tumor (Fig. 3A, Sections 1–3). Similar results were observed with the neo probe in tumors derived from pHaMAGRP.neo-transduced cells. neo expression was much enhanced at the center of the fibrosarcoma, and the overall neo level was higher than that of the background control (Fig. 3B, suggesting that the internal grp78 promoter in the retroviral vector was effective in conferring similar inducibility to the reporter gene. The neo mRNA levels in cells transduced with the SV40 internal promoter were uniformly low. Their levels were comparable to, or at best, minimally higher than that of the background control (Fig. 3C), showing low SV40 promoter activity in these tumor environments. Microscopic examination revealed that the fibrosarcomas derived from vHaMAGRP.neo and vHaMASV.neo tumors were similar in appearance (data not shown). Within the central portion of the tumors were areas of necrosis associated with polymorphonuclear leukocytes. Thus, the enhanced neo expression in these regions in the vHaMAGRP.neo tumors strongly suggests that the cells were experiencing a stress response resulting in the specific activation of the grp78 promoter but not of the SV40 promoter.

These combined results demonstrate that a 600-base pair subfragment of the grp78 promoter, as an internal promoter within a HaMSV/MRD1 retroviral vector, is able to confer high level expression of a reporter gene in a murine fibrosarcoma in vivo. Unlike other viral or cellular promoters which express constitutively in normal and malignant cells, the stress-inducible grp78 promoter offers a novel approach for targeted gene therapy for tumors in vivo. The truncated grp78 promoter described here may be useful to express a wide range of therapeutic reagents in retroviral vectors or other gene delivery systems, separating viral genes on two different plasmids. J. Virol., 62: 1120–1124, 1988.

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References

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