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Tumor-specific activity of cellular regulatory elements is down-regulated upon insertion into the herpes simplex virus genome

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Transcriptional targeting of viral genes is a promising strategy to achieve tumor-specific replication of oncolytic viruses. Due to its natural tropism, herpes simplex virus type 1 (HSV-1) may be an ideal tool for oncolytic therapy of brain tumors such as malignant glioblastoma. To study whether glioma-specific gene expression can be accomplished within the HSV-1 genome, four cellular regulatory elements were exemplarily studied. Whereas the human telomerase reverse transcriptase (hTERT) and survivin promoters and the nestin and vascular endothelial growth factor A (VEGF-A) enhancers displayed pronounced glioma specificity after plasmid transfection, only the nestin enhancer conferred a certain selectivity for glioma cells and notable activity when transferred into the viral genome. The nestin enhancer was also found to be highly useful for tumor cell-specific expression of a therapeutically relevant gene (interleukin-2) when tested in combination with the hTERT or simian virus 40 (SV40) early promoter in the HSV-1 genome. Because activity of the chosen promoter in a tumor is a prerequisite for the successful application of an oncolytic virus, we examined whether the activity of a promoter can be deduced from the amounts of cellular mRNA or protein expressed under its control. We found little correlation between promoter activity and mRNA levels of the corresponding gene, whereas protein expression was more closely related to promoter activity. We conclude that the cellular elements are differently regulated in the viral and cellular genomes. Mechanistic insight into the differential regulation is required to improve and refine the design of transcriptionally targeted HSV vectors. *Journal of NeuroVirology* (2008) **14,** 522–535.

Keywords: oncolytic herpesvirus; glioblastoma; viral gene regulation; transcriptional targeting

Introduction

Oncolytic viruses, capable of the selective destruction of tumor cells while leaving normal tissue unaffected, are considered to be a possible treatment option for malignant diseases for which conventional therapies are limited and insufficient (Aghi and Martuza, 2005; Parato *et al*, 2005). One example is malignant glioblastoma, the most aggressive of human brain tumors, which remains the most challenging in treatment. Surgery, radiation, and chemotherapy are usually without long-lasting benefits (Kleihues *et al*, 2002; Stewart, 2002) and survival is normally less than 1 year, even after

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treatment (Lacroix *et al*, 2001; Ohgaki *et al*, 2004). The striking insufficiency of conventional therapies demands the development of alternative treatment options for this fatal disease.

Oncolytic viruses based on herpes simplex virus type 1 (HSV-1) are particularly suitable for the treatment of tumors of the central nervous system (CNS) due to the natural tropism of HSV-1. Oncolytic viruses can self-replicate within cancer cells and ultimately destroy these cells. In contrast to other anticancer agents, e.g., suicide vectors, oncolytic viruses are maintained or even amplified until the tumor cells are eliminated. In clinical trials HSV mutants engineered to achieve tumor-selective replication displayed a remarkable safety profile over a wide range of virus doses (Harrow *et al*, 2004; Markert *et al*, 2000; Rampling *et al*, 2000). However, the efficacy of these oncolytic HSV for glioma therapy is not yet satisfactory. In order to reduce the replication capacity in normal cells, most of the oncolytic HSV were deleted of both copies of the neurovirulence gene γ34.5 as well as of the ribonucleotide reductase gene. These deletions resulted in preferential viral replication in tumor cells but at the same time reduced the oncolytic efficacy of the mutant viruses as compared to wild-type HSV-1. To retain tumor specificity as well as full replication capacity in the target cells, transcription of the viral gene(s) providing cell specificity can be placed under the control of a cellular glioma-specific promoter (Chung *et al*, 1999; Kambara *et al*, 2005). Thus, expression of these viral genes and the lytic viral replication cycle could be restricted to malignant glioma cells.

This strategy raises the questions of which glioma-specific promoters might be useful for transcriptional targeting of oncolytic HSV and whether the promoters will retain their specificity once they are transferred into the viral genome. To address these questions, we chose four different cellular promoters known to be active in glioma and compared their activities after plasmid transfection or in the context of the viral genome after infection of cells. The promoter of the human telomerase reverse transcriptase (hTERT) gene has been reported to be active only in embryonic and highly proliferating cells (Avilion *et al*, 1996), including approximately 80% of human glioblastomas, but not in normal glial tissue (Boldrini *et al*, 2006; Falchetti *et al*, 2002). The inhibitor of apoptosis survivin is overexpressed in cancer cells and is detected in 90% of glioblastomas (Ambrosini *et al*, 1997; Kajiwara *et al*, 2003). Both hTERT and survivin expression are regulated at the transcriptional level (Bao *et al*, 2002; Poole *et al*, 2001). Vascular endothelial growth factor A (VEGF-A) is highly active in glioma, inducing the formation of new blood vessels in this fast-growing tumor (Carmeliet and Jain, 2000; Chaudhry *et al*, 2001). The VEGF-A promoter contains a hypoxia response element (HRE), binding the hypoxia inducible transcription factor-1 (Hif-1), which facilitates promoter activity in hypoxic environments (Liang *et al*, 2002). Thus, the HRE of VEGF-A is expected to show specific activity in and around the necrotic foci of glioblastoma (Rong *et al*, 2006). The expression of the nestin gene, which encodes an intermediate neural filament found in CNS stem/progenitor cells and in CNS tumors (Lendahl *et al*, 1990), is regulated by an enhancer located in the second intron of the gene (Lothian and Lendahl, 1997). The absence of nestin enhancer activity in normal glia cells and its prominent activity in a variety of brain tumors implies that glioma-specific gene expression may be achieved through the usage of the nestin enhancer (Dahlstrand *et al*, 1992; Johe *et al*, 1996).

Therapy with a transcriptionally targeted oncolytic virus can only be successful if the cellular promoter used for transcriptional targeting is active in the tumor cells of a patient. Accordingly, rapid and clinically applicable methods will be required for determining the activity of the respective cellular promoters in the tumor biopsy of an individual patient to facilitate the choice of an appropriate oncolytic virus for treatment of this tumor. To this end, we compared the activities of four cellular regulatory elements as measured by reporter gene expression with the cellular levels of transcript and protein expressed from the corresponding genes.

We confirmed the glioma-specific activity of the cellular regulatory elements by reporter assays after plasmid transfection, but also clearly demonstrated that the same cellular elements act differently when integrated into the HSV-1 genome. Whereas the activity of a cellular promoter inserted into a plasmid-based vector may be deduced from immunohistochemical analysis of the expression of the corresponding cellular protein, the activity of such promoters within the viral genome will most likely have to be evaluated empirically.

Results

Reporter plasmids for the evaluation of glioma specificity of the cellular promoters

Transcriptional targeting of viral genes using tumor-specific cellular promoters is one strategy to establish tumor-specificity in oncolytic viruses. The aim of this study was to examine whether the tumor-specific activity of cellular promoters was retained after their insertion into the HSV-1 genome. To this end, we compared the activity of selected regulatory elements, either after transient transfection into cells or after insertion into the viral genome and infection of cells with the respective virus. The promoters of the human telomerase reverse transcriptase (hTERT) and survivin genes as well as the enhancer elements of the nestin and the vascular endothelial growth factor A (VEGF-A) genes were

examined as these genes display preferential expression in glioma cells (Boldrini *et al*, 2006; Johe *et al*, 1996; Kajiwara *et al*, 2003). The individual elements were amplified from genomic DNA by polymerase chain reaction (PCR) and cloned into firefly luciferase reporter plasmids (Figure 1). The enhancer elements were tested in connection with the minimal early promoter of simian virus 40 (SV40). A construct containing the HSV-1 Us3 promoter was generated to assess the activity of a viral promoter for comparison. All data of the firefly luciferase assays were normalized to the reporter activity of the cotransfected plasmid ph*RLuc,* expressing *Renilla* luciferase under control of the HSV-1 thymidine kinase promoter.

Transcriptional activity of the promoter and enhancer elements following transient transfection into glioma cells

The glioma cell lines U87MG, U118, A172, and U251 and primary human fibroblasts (HFs) for comparison were transfected with the reporter plasmids to assess whether the activity of the regulatory elements is restricted to tumor cells. We observed strong transcriptional activity for the hTERT (Figure 2a) and survivin (Figure 2b) promoters in three of four glioma cell lines. Both promoters were only weakly active in U87MG cells. Whereas hTERT promoter activity in these cells was comparable to that of the SV40 minimal promoter, survivin promoter activity proved to be even lower. In HFs survivin promoter activity did not exceed background level, whereas hTERT promoter activity was measurable, but below that of the SV40 promoter.

Both enhancer elements, the second intron of the nestin gene and the hypoxia response element of VEGF-A, enhanced the transcriptional activity of the SV40 minimal promoter at least fourfold in the four glioblastoma cell lines (Figure 2c, e). The SV40 promoter activity was increased up to 12-fold by the nestin enhancer (U251 cells) and 13-fold by the VEGF-A enhancer (U118 cells), whereas at best a marginal increase was observed in fibroblasts (1.3-fold). Combining the SV40 promoter and enhancer with the nestin enhancer (plasmid pNes-SVenh) further increased reporter gene activity 2- to 7-fold in glioma cells and 1.4-fold in HFs (Figure 2d). A higher activity was also observed for the VEGF-A enhancer construct pVEGF-SVenh when compared to pNes (data not shown).

The VEGF-A enhancer consists mainly of a hypoxia response element. It was therefore presumed that the activity of this enhancer would be strongest under conditions of hypoxia. To mimic such conditions, cells were treated with deferoxamine. Glioma cells subjected to deferoxamine treatment showed increased transcriptional activity of the VEGF-A enhancer ranging from 3- to 11-fold compared to that under normoxia (Figure 2f). In fibroblasts deferoxamine treatment increased VEGF-A enhancer activity 2.8-fold.

Altogether, the plasmid-based experiments indicated distinct glioma specificity of the hTERT and survivin promoters in the U118, A172, and U251 glioblastoma cells, but not in U87MG cells, whereas

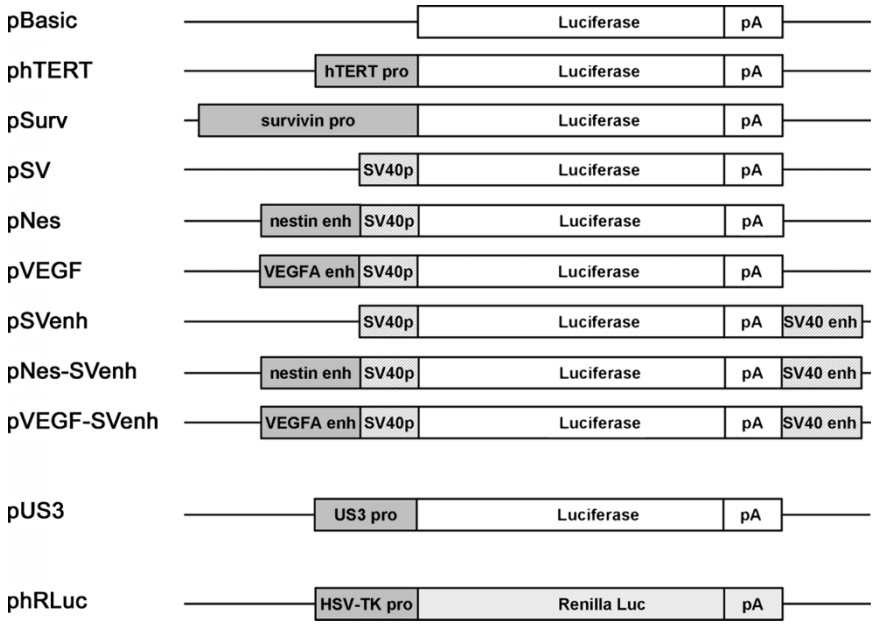

Figure 1 Schematic representation of the luciferase reporter constructs. The promoters of hTERT (phTERT), survivin (pSurv), and HSV-1 Us3 (pUS3) directly controlled the expression of firefly luciferase, whereas the activities of the enhancers of nestin (pNes) and VEGF-A (pVEGF) were evaluated in combination with the SV40 minimal promoter. Plasmids pBasic and pSV served as controls and ph*RLuc* was used to normalize for transfection efficiency.

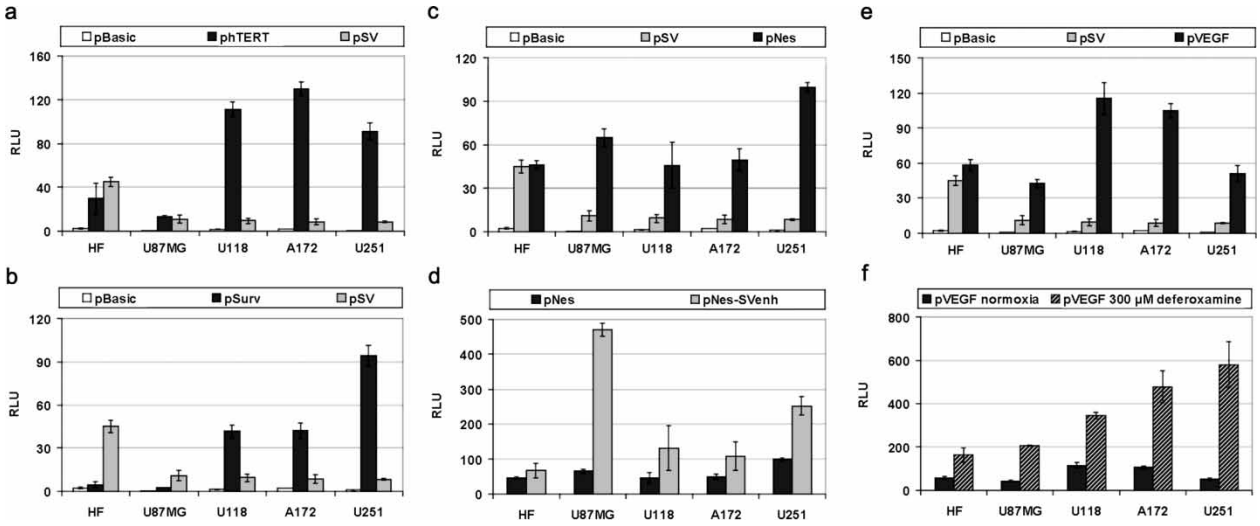

Figure 2 Activity of the promoters/enhancers in the plasmid context. Human glioma cell lines U87MG, U118, A172, and U251 and human foreskin fibroblasts (HF) were cotransfected with the indicated luciferase constructs and ph*RLuc*. The transfection experiments were done in triplicate. The activities of the hTERT promoter (**a**), the survivin promoter (**b**), the nestin enhancer (**c**, **d**), and the VEGF-A enhancer (**e**) were determined 24 h post transfection. All transfection experiments were performed simultaneously, but for the sake of clarity the results for the individual plasmids are depicted separately in comparison to the activities of the control plasmids. To compare the activities of the VEGF-A enhancer under normoxic and hypoxic conditions, cells were incubated in the absence or presence of deferoxamine (300 µM) and assayed at 24 h post transfection (**f**). A representative result of one of three independent experiments is shown in each case. Plasmid abbreviations correspond to those given in Figure 1.

the enhancer elements of the nestin and VEGF-A genes showed tumor-associated activity in all four glioma cell lines. Our data suggested that the examined regulatory elements would be suitable for the construction of transcriptionally regulated herpes simplex viruses.

Comparison of reporter activity with the cellular level of gene-specific transcripts

When considering the therapeutic application of an oncolytic HSV-1 exhibiting restricted replication capacity due to control by a tumor-specific cellular promoter element, it will be necessary to evaluate the activity of that element in the tumor tissue of the patient. Considering the heterogeneity of malignant gliomas (Liang *et al*, 2005; Sallinen *et al*, 2000), the therapeutic success of oncolytic viruses will most likely depend on the selection of the most effective viral vector from a panel of viruses with different properties. Due to the complexity of reporter gene assays, these methods can hardly be used routinely in clinical facilities and therefore a more rapid and clinically applicable approach to assess promoter activities in tumor biopsies is desirable. Quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR) would fulfill these requirements as the amounts of transcripts should reflect the activities of the respective promoters and could be determined directly from a biopsy sample.

To test the suitability of quantitative real-time RT-PCR for this purpose, we measured the relative amounts of hTERT, survivin, nestin, and VEGF-A transcripts in glioma cell lines and for comparison in human fibroblasts. The experiment was performed both with half-confluent and confluent cultures to detect a potential dependence of the transcript levels on the cell cycle. In both half-confluent (data not shown) and confluent cell layers (Figure 3a), prominent amounts of survivin mRNA were apparent, even in HF cells. These results were not in agreement with the findings of the reporter assays for the survivin promoter plasmid (see Figure 2b). Whereas characterization by melting curve analysis (data not shown) and gel electrophoresis (Figure 3b) confirmed the identity of the PCR product, immunoblot analysis with an anti-survivin antibody suggested a roughly even expression profile of the survivin protein in the examined glioma cell lines and a lack of expression in HF cells (Figure 3c). The latter result was in accordance with the result of the reporter gene assay in fibroblasts but in clear contrast to our real-time PCR data (Figure 2b). Because the transcripts of survivin and of the effector protease receptor-1 (epr-1) may originate from opposite DNA strands of the same genomic region (Ambrosini *et al*, 1997), we must conclude that the widely used RT-PCR method that we adopted here is not suitable for estimating specifically the copy number of survivin transcripts.

Accordingly, for further analysis we considered only the results obtained for hTERT, nestin, and VEGF-A mRNA expression (Figure 3d) and excluded the data for survivin. All glioma cell lines, especially lines U87MG and A172, contained considerable amounts of VEGF-A mRNA, but there was no linear relation between the mRNA amounts and

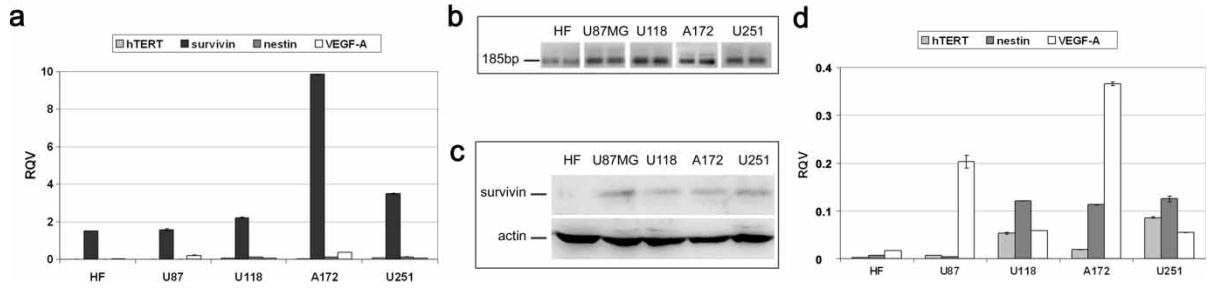

Figure 3 Real-time RT-PCR analysis of mRNA levels. (**a**, **d**) Real-time quantitative PCR. Transcript levels of hTERT, nestin, survivin, and VEGF-A were determined in duplicate. mRNA amounts relative to the amount of ABL transcripts (RQV, relative quantitation value) are plotted. The data for survivin were not considered in d. (**b**) Analysis of the survivin PCR product. PCR products amplified with primers specific for the survivin transcript were separated by electrophoresis on an 1% agarose gel to verify the correct product size of 185 bp. (**c**) Analysis of survivin protein expression. Whole-cell lysates were subjected to immunoblotting using a specific antiserum detecting survivin at 16.5 kDa. The membrane was reprobed with an actin-specific antibody.

the activties determined with the pVEGF plasmid. However, a correlation of VEGF-A transcript amounts with the activity of the VEGF-A enhancer was not expected as the VEGF-A gene promoter is physiologically active in most cells and real-time PCR reflects total promoter activity rather than enhancer activity (Yancopoulos *et al*, 2000). Transcript levels of hTERT and nestin were in reasonable accordance with the results of the reporter gene assays obtained in the glioma cell lines apart from U87MG. In this cell line we detected only minor amounts of the nestin transcript, both in confluent as well as half-confluent cells. Overall, quantitative real-time PCR data displayed limited correlation to the results obtained in the plasmid luciferase assays.

Protein expression determined by immunohistochemical staining is in line with reporter activity

Histological examination is a routine technique applied to tumor biopsies. We therefore asked whether immunohistochemical staining of glioma cells with antibodies against the proteins hTERT, survivin, nestin, and VEGF-A would be a fast and effective technique to assess the expression strength of the respective genes. Both immunohistochemistry (Figure 4a) and immunofluorescence microscopy (Figure 4b) allowed a semiquantitative evaluation of the expression levels of the analyzed proteins. Fibroblasts did not demonstrate any immunoreactivity and the lower amounts of hTERT and survivin seen in the U87MG cells are in good

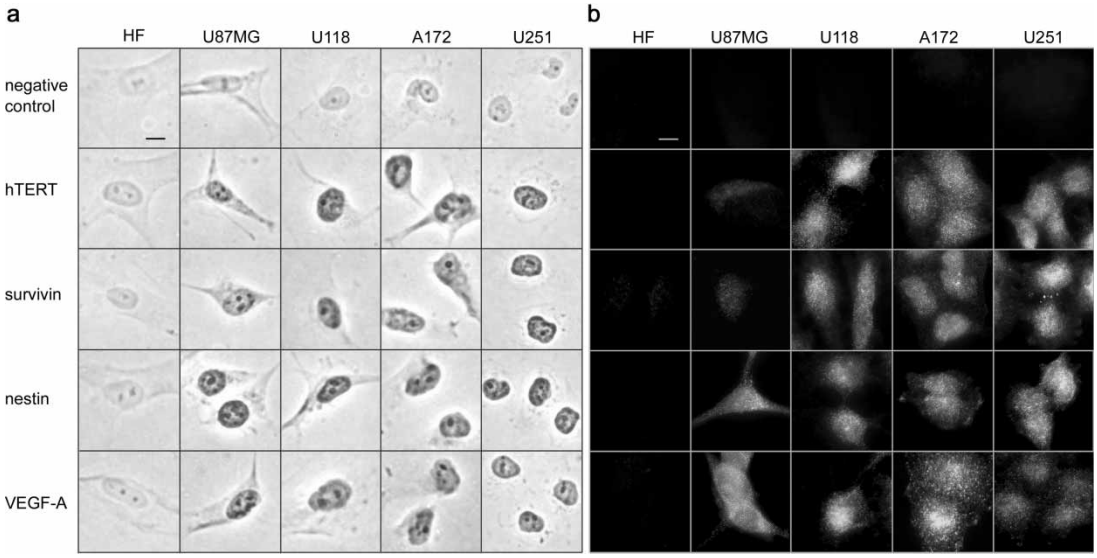

Figure 4 Immunohistochemical analysis of hTERT, nestin, survivin, and VEGF-A expression. For detection of the proteins in human fibroblasts (HF) and in the human glioma cell lines U87MG, U118, A172, and U251, specific primary antibodies were used followed either by staining with diaminobenzidine (**a**) or by incubation with a fluorescent secondary antibody (**b**). Scale bar = 10 μm.

agreement with the low luciferase activities of the corresponding promoter constructs (Figure 2a and b). In a clinical setting immunohistochemical staining may therefore be a practical method to estimate the activity of the respective gene promoters in tumor tissues.

Construction of recombinant viruses for the evaluation of tumor-specific promoter elements in the viral context

Following the insertion of a cellular promoter into a viral genome the different genetic context, e.g., differences in the surrounding chromatin structure, may cause the promoter to perform differently. Therefore, we regarded it as necessary to confirm promoter activities determined in plasmids in the context of the viral genome. We designed a strategy that allowed us to compare the activities of cellular promoter elements after transient transfection of reporter plasmids with their activities after infection of cells with recombinant viruses containing the same regulatory elements. To insert the various promoter luciferase constructs into the HSV-1 genome, we employed a recombinase FLP-mediated recombination strategy. The promoter and enhancer luciferase cassettes were subcloned into a shuttle plasmid containing a FLP recombination target (FRT) site. FLP-mediated recombination in *Escherichia coli* between this FRT site and an identical site engineered into the bacterial artificial chromosome (BAC)-cloned genome of the HSV-1 strain F (HSV-FRT BAC) resulted in the integration of the shuttle plasmid into a noncoding region between the open reading frames UL55 and UL56 of the HSV genome (Figure 5a). The resulting BACs were characterized by restriction analysis using *Bgl* II. Depending on the size of the promoter/enhancer luciferase cassette, a shift of an 8.2-kb fragment to approximately 12 kb indicated stable integration and successful generation of the recombinant genomes (Figure 5b). Recombinant viruses were generated by transfection of the recombinant BACs into Vero cells and the viral genomes characterized again by restriction analysis (data not shown).

Activity of the tumor-specific promoter/enhancer elements following integration into the HSV-1 genome

To investigate the influence of the viral context on the transcriptional activity of the regulatory elements, reporter gene assays were performed following infection of cells with the HSV-1 mutants. The absolute luciferase activities that we obtained after infection of cells were up to three orders of magnitude higher than those found with the plasmid-based assays, because unlike the plasmid vectors, the virus genomes were introduced into almost every cell upon infection and, in addition, their copy number increased rapidly within the 24 h post infection (p.i.). However, the activities achieved

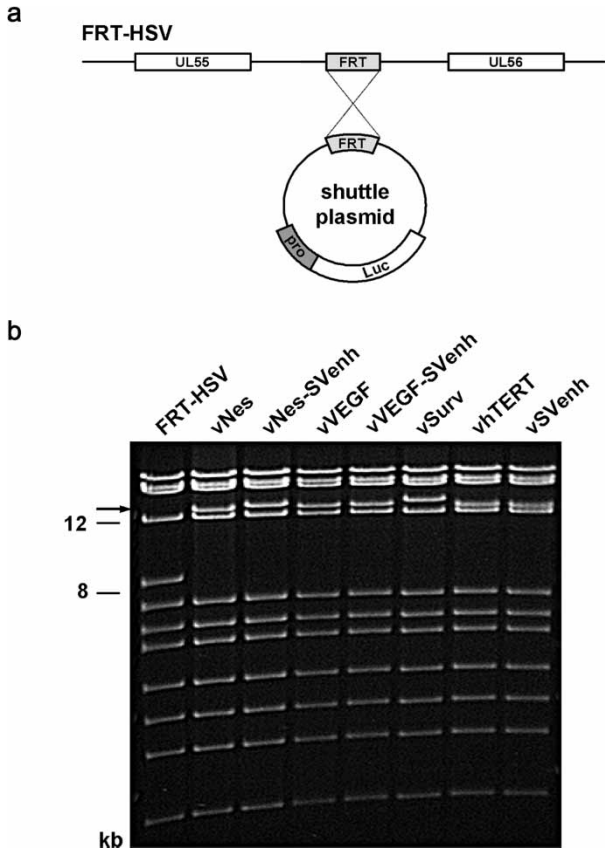

Figure 5 Construction and structural analysis of recombinant HSV-1 genomes. (**a**) HSV-1 genomes carrying the promoter luciferase constructs were generated in *E. coli* by recombinase FLP-mediated insertion of shuttle plasmids into the BAC-cloned HSV-1 genome. (**b**) DNA of the recombinant HSV1-BACs was subjected to *Bgl* II digestion and separated by electrophoresis on a 0.5% agarose gel for 16 h. Successful insertion led to a bandshift of an 8.2-kb *Bgl* II fragment to approximately 12 kb (*arrow*) depending on the size of the inserted sequences.

with viruses expressing luciferase under control of the hTERT (Figure 6a) or survivin promoters (data not shown) were rather low and at best marginally higher than the activities obtained with a virus that lacked a promoter in front of the luciferase open reading frame (ORF). In normal fibroblasts the transcriptional activity of hTERT (Figure 6a) and survivin promoters (data not shown) did not exceed background level. The virus containing the VEGF enhancer led to an activity 2.2- to 3.7-fold above background, but this was not glioma-specific as a 2.2-fold higher activity was also observed in fibroblasts (Figure 6a). Under hypoxic conditions the activity was either unchanged (U118 cells) or reduced by a factor of 1.2 to 2.2 in glioma cells. In fibroblasts the reduction was 3.2-fold, suppressing the activity under these conditions below the background level. This result suggested that the VEGF enhancer is regulated in a completely different manner when inserted into the HSV genome. The nestin enhancer element in combination with the

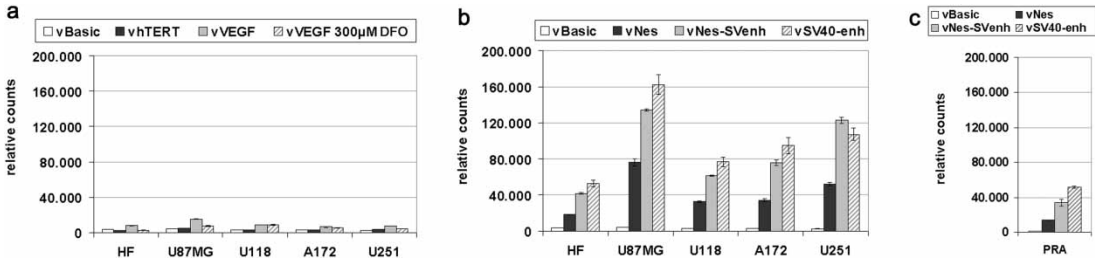

Figure 6 Activity of the cellular promoters/enhancers in the context of the HSV-1 virus genome. Human glioma cell lines, foreskin fibroblasts (HF) and primary rat astrocytes (PRA) were coinfected with the virus carrying the indicated promoter/enhancer–firefly luciferase cassette (MOI = 0.02) and the virus carrying the ph*RLuc* cassette (MOI = 0.01). All infections were performed in triplicate. (**a**) Activities obtained after infection with the viruses carrying the hTERT promoter or the VEGF enhancer under normoxic and hypoxic conditions (300 µM deferoxamine [DFO]). The activities following infection of the indicated cells (**b**) and of primary rat astrocytes (**c**) with the viruses carrying the nestin enhancer are shown in comparison to activities obtained after infection with the control virus. All values were normalized to the *Renilla* luciferase activities resulting from the co-infecting *hRLuc*-virus. (a and b) One representative result of three independent experiments is shown.

SV40 promoter (vNes) displayed the highest activity of all four cellular elements, with an activity fivefold above background in fibroblasts and 11- to 21-fold in glioma cells. In summary, the activity of the nestin enhancer was 1.7- (U118) to 4.2- (U87MG) fold higher in glioma cells than in fibroblasts (Figure 6b), indicating a certain selectivity of this regulatory element for glioma cells. As observed in the plasmid assays, the nestin enhancer plus the SV40 enhancer (vNes-SVenh) was more effective than the nestin enhancer alone (Figure 6b), but the enhancement was 1.7- to 2.3-fold only. Remarkably, the SV40 promoter either alone (data not shown) or in combination with its enhancer (Figure 6b) displayed an even slightly higher activity (1.1- to 2.1-fold and 1.1- to 1.3-fold, respectively), suggesting that the nestin enhancer rather led to a reduction than an increase of the SV40 promoter acitivity in the viral context. Some of the viruses were also tested in primary rat astrocytes (Figure 6c). The activities of the promoters found in these cells closely resembled those observed in the primary fibroblasts, suggesting that the latter cells can be used as a relevant control.

The results of the virus infection experiment revealed a low activity of three of the four examined cellular elements when inserted into the viral genome. Only the nestin enhancer displayed a notable activity in glioma cells, but the activity in fibroblasts and primary astrocytes, although lower, was not negligible. In the viral context, the SV40 regulatory sequences showed the highest activity and thus behaved completely different than in the transfection assays. We conclude that the plasmid-based assay results do not reflect the activity of the promoter element when transferred into the viral genome, enforcing the necessity of evaluating promoter elements for the construction of oncolytic viruses within the context of the virus rather than within a plasmid sequence.

Activity of a viral promoter in the plasmid and viral context

For construction of a transcriptionally regulated HSV, a viral promoter has to be replaced by a suitable cellular promoter. Thus, we considered it worthwhile to rate the activity of a viral promoter in comparison to that of the cellular regulatory elements—both after plasmid transfection as well as virus infection. As an example, we chose the Us3 promoter of HSV-1. In normal fibroblasts and U87MG glioma cells, the activity of the Us3 promoter in the plasmid context was close to the activities of the cellular regulatory elements, whereas in the glioma cell lines U118, A172, and U251, the viral promoter activity was severalfold lower (range 2.5- to 20.3-fold) (Figure 7a). In contrast, when integrated into the viral genome, the Us3 promoter activity was considerably higher as compared to the cellular regulatory elements in HF and all four glioma cell lines tested (Figure 7b). Although 3.8 to 8.8-fold weaker than the Us3 promoter, the activity of the nestin enhancer in the glioma cells was at least in a range that may qualify it for the construction of a transcriptionally targeted HSV. Again, the rather contrary properties of the cellular and viral promoters in the plasmid and viral context underline the need for evaluation of cellular promoters in the viral genome.

Tumor cell–specific expression of a cytokine

Because our results suggested that the nestin enhancer element is suitable for glioma cell–specific transcriptional targeting, we tested whether this element can be used to control the expression of a gene with therapeutic potential. We chose interleukin-2 (IL-2), because this cytokine can activate tumor-infiltrating lymphocytes, which may then complement the activity of the oncolytic virus. A combination of IL-2 and HSV-TK gene therapy has already been evaluated in a clinical trial in patients

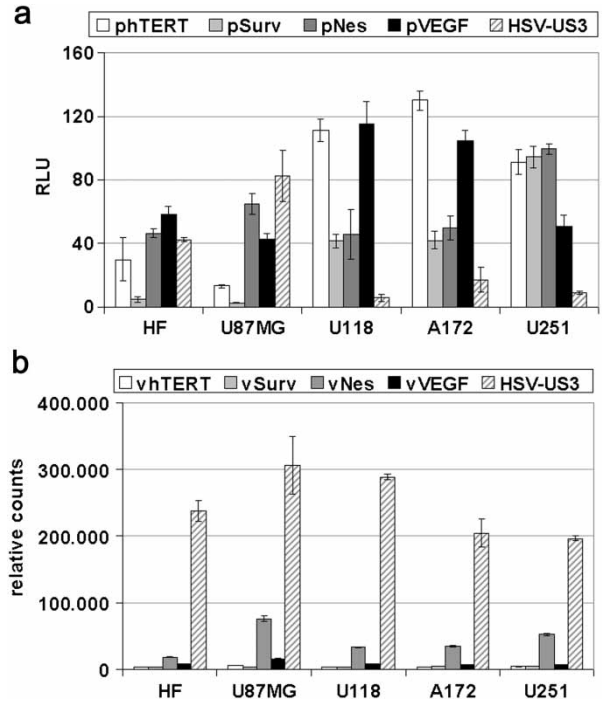

Figure 7 Relative activity of the HSV-1 Us3 promoter. Shown are the relative activity values determined for the Us3 promoter construct (**a**) and the Us3 promoter luciferase virus (**b**) in comparison to the results obtained in the plasmid transfection and virus infection experiments.

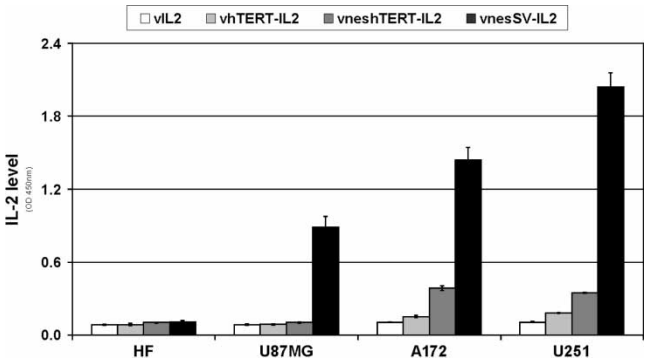

Figure 8 Tumor cell–specific expression of interleukin-2. The indicated cells were infected with HSV-1 recombinants carrying the ORF mIL-2 gene without a promoter (vIL2), with the hTERT promoter (vhTERT-IL2), the nestin enhancer combined with the hTERT promoter (vneshTERT-IL2), or the nestin enhancer combined with the SV40 minimal promoter (vnesSV-IL2). All infections were performed in triplicate. The levels of mIL-2 in the cell culture medium were determined by ELISA at 48 h p.i.

with recurrent glioblastoma (Colombo *et al,* 2005). Restricting the expression of the cytokine to the tumor and immediate adjacent areas would most likely be highly beneficial. HSV-1 recombinants were constructed that contained either the murine IL-2 ORF alone or in combination with the hTERT promoter, the nestin enhancer plus the hTERT promoter, and the nestin enhancer plus the SV40 promoter, respectively. Following infection with the viruses carrying the hTERT promoter or no promoter in front of the mIL-2 ORF almost no mIL-2 expression was observed (Figure 8), irrespective which cells were infected. This is in agreement with the corresponding results of the luciferase experiments (Figure 6a), indicating that the activity of the hTERT promoter is rather low in the glioma cell lines. Next, we evaluated the nestin enhancer, either in combination with the hTERT promoter or with the SV40 minimal promoter. The nestin enhancer increased the expression of mIL-2 in both combinations in a tumor cell–specific manner, i.e., expression in the primary cells was negligible. The combination with the hTERT promoter may lead to an even higher specificity than that seen with the SV40 minimal promoter, because in U87MG, which displayed a low hTERT promoter activity (Figure 2a, 4), no mIL-2 expression was observed. However, combination of the nestin enhancer with the SV40 promoter resulted in a higher mIL-2 production, suggesting that this promoter as a viral element may be recognized with some preference in the context of the HSV-1 genome. The results underline that the nestin enhancer element is highly useful for expression of genes of therapeutic relevance.

Discussion

Transcriptional targeting is considered to be a promising approach for construction of oncolytic HSV vectors with specificity for malignant glioma. In this study four cellular promoters with expected activities in glioma cells were evaluated for their suitability in driving the expression of HSV genes. All four regulatory elements displayed tumor specificity in at least three of four glioma cell lines when tested in reporter assays after plasmid transfection. Unexpectedly, when the regulatory elements were transferred into the HSV-1 genome and their activities were tested after infection of cells, their expression strengths in the glioma cells were usually not much higher than in untransformed human fibroblasts and, overall, the promoter strengths were low in comparison to that of a viral promoter. The only exception was the nestin enhancer in that its activity in the glioma cells was considerably higher than in human fibroblasts and its strength, in comparison to a viral promoter, was at least in a range that may qualify it for transcriptional targeting of viral or therapeutic gene expression.

When tested after transfection, the promoters of hTERT and survivin displayed high activities in three of four glioma cell lines, whereas in human fibroblasts their activity was either lower than that of the SV40 minimal promoter (hTERT) or almost absent (survivin). In U87MG glioma cells both promoters showed only weak activity, underscoring that cells derived from different gliomas exhibit

various biological features. Thus, not every glioma-specific promoter will work in every glioma type, underlining the need to characterize tumor biopsies in more detail in order to find the most useful tools for therapy. U87MG cells retain the wild-type version of p53 (Asai *et al*, 1994), which is known to negatively regulate the hTERT and survivin promoters, explaining their low activities in this cell line (Mirza *et al*, 2002; Xu *et al*, 2000). For comparison, we chose human fibroblasts as a convenient source of primary nontransformed cells, being aware of the limitations of this cell system. For example, *in situ* most normal cells surrounding a tumor are probably in G0 phase whereas the fibroblasts in culture were growing, although slowly. This may explain the low activity of the hTERT promoter in HFs, as was described before (Masutomi *et al*, 2003). Also, the activity of the minimal SV40 promoter used for comparison was often higher in HFs than in the glioma cell lines, implying that the differential activity of the glioma-specific promoters may even be more pronounced *in vivo*. Notably, the results obtained in the fibroblasts and in primary astrocytes were comparable. Differences exist also between primary glioblastoma multiforme tumor samples and established gliobastoma cell lines, as has been confirmed recently (Vogel *et al*, 2005). We are convinced, however, that human glioblastoma cell lines provide a highly suitable and convenient system for the initial investigation of the principles of transcriptional targeting of HSV.

Both the nestin and the VEGF enhancers conferred preferential expression of the reporter gene in most glioma cell lines, especially when comparing the activities to those of the minimal promoter. As expected, the VEGF enhancer reached its highest activity under hypoxic conditions. Remarkably, the activity of the nestin enhancer could be further strengthened in combination with the SV40 enhancer. This observation indicates that the nestin enhancer controls primarily cell type specificity and not necessarily strength of expression. Accordingly, construction of hybrid promoters may be an option. Such an approach has been used for enhancing the activity of the hTERT promoter in the context of an adenovirus vector (Song, 2004). The challenge will be to arrange the regulatory elements in such a way that strong expression is ensured and cell type specificity is retained.

Determining the activity of tumor-specific promoters in tumor biopsies will be a prerequisite for the successful application of transcriptionally targeted oncolytic viruses. Although microarray analysis may become a routine technique for diagnostic purposes in the future, it is not clear whether mRNA amounts reflect the strength of a promoter. Our data did not indicate a linear correlation between the levels of mRNA and the activities of the corresponding promoters. Remarkably and in agreement with the results of others (Kurihara *et al*, 2000), we found very little nestin mRNA in U87MG cells, yet the activity of the nestin enhancer was even higher in this cell line than in U118 and A172 glioma cells, which contained more than 10-fold higher amounts of nestin mRNA. Despite the low amount of mRNA, we observed good expression of the nestin protein in the U87MG cells. High protein expression of nestin in this cell line was also reported by Rutka *et al* (1999) and Strojnik *et al* (2006). Thus, there does not seem to be a linear correlation between promoter strength and mRNA levels or between the amounts of nestin mRNA and protein. In addition, precise quantitation of mRNA amounts may sometimes be obscured by binding of primers to transcripts that display partial homology to the target sequences or to the antisense strand, as may be the case for survivin (Ambrosini *et al*, 1997; Zaman and Conway, 2000). In short, RNA levels do not seem to be a good predictor of promoter strength. However, the lower amounts of hTERT and survivin proteins observed in the U87MG cells as compared to the other glioma cell lines and the lack of immunoreactivity in fibroblasts were in good agreement with the activities of the corresponding promoters in these cells. Further investigations are needed to learn whether this observation can be confirmed in other cells and for other relevant genes. If this is the case, histochemical staining, a routine technique for characterization of tumors, could be easily adopted to deduce the activity of tumor-specific promoters.

When the cellular regulatory elements were inserted into the viral genome, they behaved almost completely differently than in the plasmid context. The activities of most of the elements were rather low and often slightly exceeded the background activity of the promoterless control construct only. Even the activity of the nestin enhancer was in a moderate range when compared to a viral promoter and we observed only a limited selectivity of the luciferase expression in glioma cells. An explanation for this behavior of the cellular promoters may come from the comparison of the activity of the viral Us3 promoter after plasmid transfection and after infection. Following plasmid transfection the Us3 promoter was weakly active in three of four glioma cell lines and displayed at best a similar activity in HFs and U87MG cells as compared to the cellular regulatory elements. In the viral context, however, its activity was way beyond that of the cellular promoters, indicating that the viral promoter is positively regulated by viral factors whereas the cellular promoters are most likely not. As HSV-1 infection leads to a shutdown of host protein synthesis (Roizman, 2001), it may even repress the transcription of cellular genes or counteract the activity of cellular transcription factors, explaining the low activity of the cellular elements. At present, it remains unclear why the nestin enhancer showed a higher activity and selectivity for glioma cells

when inserted into the viral genome as compared to the other cellular promoters. The SV40 promoter as a viral element, which was used in combination with the nestin enhancer, probably bears some resemblance to an HSV-1 promoter and may have come under the control of the HSV-1 regulatory proteins, further enhancing the activity. A detailed study of the binding and activity of cellular and viral transcription factors would be needed to gain insight into the complex regulation of the promoter and enhancer elements when embedded into the HSV-1 genome. Because we cannot exclude activity of the SV40 promoter in the HSV-1 context in normal cells, we combined the tumor cell–specific hTERT promoter with the nestin enhancer for expression of a therapeutically relevant protein (interleukin-2). IL-2 has been shown to function as a potent antitumor agent by stimulating cytotoxic T lymphocytes and enhancing the activity of natural killer (NK) cells, lymphocytes that were observed to infiltrate into tumor tissues. Virus-mediated expression of mIL-2 has been shown to effectively inhibit tumor growth in a subcutaneous glioma mouse model, even when expressed at relatively low levels (Chen *et al*, 2001). We confirmed that expression of IL-2 by the virus occurred only in cells in which both the nestin enhancer and the hTERT enhancer promoter were active. Thus, the combination with the hTERT promoter may represent a new tool in transcriptional targeting of oncolytic viruses to malignant glioma. In addition, this combination can be used to achieve highly glioma cell specific, and therefore particularly safe, expression of target genes at an adequate therapeutic level.

Because there are different classes of viral promoters in HSV-1 that may vary in their properties, the question arises which viral promoters will be suitable for replacement with cellular elements. Immediate-early (IE) promoters are activated by cellular transcription factors and viral factors brought along with the virion into the infected cell. Therefore, IE genes are good candidates for transcriptional targeting. However, because the regulatory proteins encoded by IE genes may be needed in substantial quantity throughout the infection cycle, it remains to be tested whether tumor-specific promoters can provide and maintain adequate expression. Early and late promoters are mainly controlled by viral transcription factors and late structural proteins are often needed in great abundance disqualifying them for replacement with tumor-specific promoters. We consider accessory genes encoding proteins that are nonessential for replication and packaging of the viral genome and that are probably needed in lower quantity only as most promising candidates for transcriptional targeting. Such genes, e.g., the γ34.5 gene of HSV-1, can be decisive for the replication capacity of HSV in certain cell types and, indeed, the γ34.5 gene has been successfully replaced by tumor-specific promoters (Chung *et al*, 1999; Kambara *et al*, 2005). As an example of this class of viral genes, we chose Us3, which plays a role in the egress of virus particles from the nucleus as well as in apoptosis inhibition (Leopardi *et al*, 1997). Us3 expression driven by a tumor-specific promoter may still provide a selective replication advantage to a HSV-1 mutant even if expression is below that of a wild-type virus.

The results of our study let us conclude that the regulation of promoters inserted into the viral genome is driven by mechanisms different from those that control promoters in the cellular chromatin. In order to enable the rational design of transcriptionally targeted HSV, it seems mandatory to investigate how cellular promoters integrated in the viral genome are recognized by their specific cellular transcription factors and how viral factors interfere with and potentially impair the function of the cellular transcription factors and the regulation of the promoters. In the absence of this knowledge, it seems necessary to evaluate each tumor-specific promoter individually for its suitability for transcriptional targeting after its integration into the HSV-1 genome.

Materials and methods

Cells and viruses

Human glioblastoma cell lines U87MG, U118, A172, and U251 were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and human foreskin fibroblasts (HFs), HeLa cells, and Vero cells in DMEM containing 5% FCS. Media were supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. The parental HSV strain used in this study is derived from the BAC-cloned HSV-1(F) genome as described elsewhere (Tanaka *et al*, 2003a). Reconstitution of virus, preparation of virus stocks and viral DNA, and determination of virus titers were done on Vero cells essentially as described previously (Dohner *et al*, 2002).

Culture of primary glial cells

Primary cultures of glial cells were prepared from neonatal Sprague-Dawley rat cerebra as described (McCarthy and de Vellis, 1980; Stangel and Bernard, 2003). Brains were freed from meninges, and dissociated mechanically and enzymatically with trypsin and DNAse (both from Sigma-Aldrich, Deisenhofen, Germany). Cells were cultured in DMEM supplemented with 10% heat inactivated FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin. Medium was changed on the next day, and after

several days a confluent monolayer of astrocytes developed.

Plasmid construction

Human genomic DNA from HeLa cells was used as a template for PCR amplification of the promoter and enhancer elements using the following primers: hTERTp.for (5'-GCC AGA TCT GGC CCC TCC CTC GGG TTA C-3′), hTERTp.rev2 (5′-GCC AAG CTT AGG GCT TCC CAC GTG CGC AG-3′), nestp.for (5′-GCC TCG AGA CTG CTT AGA GAT CCT G-3′), nestp.rev (5′-GCC AGA TCT ACA CAC ACC CAC ACA AG-3′), vegfa.for (5′-GCC TCG AGA AGT AGC CAA GGG AT-3′), and vegfa.rev (5′-GCC AGA TCT AGC TGT GTG GTT CCG GGG T-3′). The cytomegalovirus (CMV) promoter was amplified by PCR from pECFP-N1 (Clontech, Mountain View, CA) using primers cmvp.for (5′-GCC GGA TCC TAG TTA TTA ATA GTA ATC AAT-3′) and cmvp.rev (5′-GCC AAG CTT GAT CTG ACG GTT CAC TAA CCA-3′). The HSV-1 Us3 promoter was PCR amplified from HSV-1 strain F BAC DNA using primers US3p.for (5′-GCC GAA TCC GAT CGG GCG GGT GGG TTT GGG TAA GTC-3′) and US3p.rev (5′-GCC AAG CTT GCG CGC ACC GTG AGT GCC AAC CAA C-3′). PCR products were verified by DNA sequencing, treated with *Bgl* II, *Hind* III, *Mlu* I, and *Bam* HI, respectively, and cloned into the reporter plasmids pGL3B, pGL3C, or pGL3P (Invitrogen, Carlsbad, CA). A pGL3B-based plasmid containing the survivin promoter was kindly provided by M. Murphy (Hoffman *et al*, 2002). The pGL4.74*hRLuc* control plasmid was obtained from Promega (Madison, WI). For construction of the interleukin plasmids, the hTERT promoter was amplified from pGL3B-hTERT using primers pT3tert.for (5′-GCC GCG GCC GCT GGC CCC TCC CTC GGG TTA-3′) and pT3tert.rev (5′-GCC ACT AGT AGG GCT TCC CAC GTG CGC A-3′), the nestin enhancer plus SV40 minimal promoter was amplified from pNes-SVenh using primers pT3nestert.for (5′-GCC GCG GCC GCA GTG CTT AGA GAT CCT GAG A-3′) and pT3nesMP.rev (5′-GCC ACT AGT AAG CTT TTT GCA AAG CCT A-3′) and the nestin enhancer plus hTERT promoter was amplified from phTERTnes using primers pT3nestert.for and pT3tert.rev. Plasmid phTERTnes was constructed by amplifying the nestin enhancer from pNes-SVenh using primers nestp.for and nestp.rev, cloning of the PCR fragment first into plasmid *pEGFP-C1* via MluI and BamHI and finally into *phTERT* via MluI and BsmI. The promoter elements were cloned in front of the mIL-2 ORF in the plasmid *pT3_IL-12_4-1BBL_IL-2* (kindly provided by Frank Schnieders, Provecs Medical GmbH, Hamburg) using NotI and SpeI sites. The promoter mIL-2 cassettes were then transferred into *pOri6K-Kan1* via *MluI/NotI* and *MluI/SpeI* (mIL-2 without promoter), respectively.

BAC mutagenesis

A HSV1-BAC carrying a FRT site between genes UL55 and UL56 was generated as follows: Plasmid pGP704-Kan (Borst and Messerle, 2005) was used as a template for PCR amplification of a kanamycin resistance gene flanked by two FRT sites. The primers carried 42 and 57 bp long overhangs at their 5′ ends, which displayed homology to the intended integration site between UL55 and UL56. *E. coli* DH10B containing the HSV-1 BAC and plasmid pKD46, providing the genes for the bacteriophage λ red recombination functions upon induction with arabinose (Datsenko and Wanner, 2000), were transformed with the PCR product and bacterial clones harboring recombinant BACs were selected by incubation at 43°C on agar plates containing 17 μg/ml of chloramphenicol and 25 μg/ml of kanamycin. Excision of the resistance cassette resulting in a single remaining FRT site was achieved by transiently expressing recombinase Flp from plasmid pCP20 as described previously (Cherepanov and Wackernagel, 1995). Successful recombination was verified by restriction analysis of BAC DNA.

HSV-1 BACs carrying different promoter elements in front of the firefly luciferase ORF were constructed by recombinase FLP-mediated insertion of shuttle plasmids based on the oriR6Kγ replicon into the HSV-1-FRT-BAC as described previously (Borst and Messerle, 2005). In brief, the promoter/enhancer-luciferase cassettes were excised from pGL3 constructs and subcloned into a derivative of the shuttle vector pOri6K-Kan1, which carries a kanamycin resistance gene adjacent to a single FRT site and a polylinker. *E. coli* DH10B containing the HSV-1-FRT-BAC were transformed with pCP20 and incubated at 30°C on agar plates containing 17 μg/ml of chloramphenicol and 50 μg/ml of ampicillin. Bacteria containing both pCP20 and HSV-1-FRT-BAC were then transformed with the respective pOri6K-Kan1 construct and clones harboring recombinant BACs were selected by incubation at 43°C on agar plates containing 17 μg/ml of chloramphenicol and 25 μg/ml of kanamycin. Construction of the HSV-1 BACs carrying the mIL-2 ORF under control of the different promoters was done in an analogous manner.

Reporter gene assays

Transfection of reporter plasmids was performed by the calcium phosphate method. Cells were plated in 12-well tissue culture dishes at a density of 5×10^4 cells/well (HFs, U87MG, A172) or 1×10^5 cells/well (U118, U251, primary astrocytes). Twenty-four hours later the medium was changed and where indicated supplemented with 300 μM deferoxamine. After 2 h cells were transfected according to the manufacturer's protocol (Calcium Phosphate Transfection Kit; Sigma-Aldrich, St. Louis, MO) using 2 μg of the respective firefly luciferase constructs and

1 μg of the *Renilla* luciferase construct pGL4.74h*RLuc* for normalization. After incubation for 16 h, the medium was replaced and the cells were incubated for another 8 h, followed by cell lysate preparation and estimation of luciferase activity using the Dual-Luciferase Reporter System Assay kit according to the manufacturer's protocol (Promega, Madison, WI) and a 96-well plate reader.

Viral infection was performed as follows: cells were plated in 12-well tissue culture dishes as above. After 24 h the medium was taken off and a viral dose corresponding to an maultipicity of infection (MOI) of 0.02 for the firefly luciferase viruses and to an MOI of 0.01 for the *Renilla* luciferase control virus was added. Following centrifugation at $750 \times g$ for 15 min, medium was added. Deferoxamine at a final concentration of 300 μM was added where indicated. Twenty-four hours after infection cell lysates were prepared and luciferase activity was estimated as above.

Relative light units were calculated by normalizing firefly luciferase activity to the *Renilla* luciferase activity obtained from the cotransfected control plasmid (10^5 units) or coinfection with the control virus (10^7 units).

Quantitative real-time RT-PCR

Cytoplasmic RNA was isolated from glioma cell lines and human foreskin fibroblasts with the RNeasy mini kit (Qiagen, Hilden, Germany). The RNA was DNase treated with TURBO DNA-free (Ambion, Austin, TX) and 1 μg RNA was reverse transcribed using oligo-dT primers and Thermoscript reverse transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's protocols. Specific cDNAs were amplified by PCR using primers hTERT-rt.for (5′-TGA CAC CTC ACC TCA CCC AC-3′), hTERT-rt.rev (5′-CAC TGT CTT CCG CAA GTT CAC-3′), surv-rt.for (5′-AAG AAC TGG CCC TTC TTG GA-3′; specific for exon 2), surv-rt.rev (5′-CAA CCG GAC GAA TGC TTT TT-3′; specific for exon 3), nestin-rt.for (5′-AGC TGG CGC ACC TCA AGA TGT-3′), nestin-rt.rev (5′-CTG AAA GCT GAG GGA AGT CTT-3′), vegfa-rt.for (5′-TAC CTC CAC CAT GCC AAG TG-3′), vegfa-rt.rev (5′-TAG CTG CGC TGA TAG ACA TCC A-3′), ABL-rt.for (5′-ACG TGC CTG AGA TGC CTC ACT-3′), ABL-rt.rev (5′-AAG GCG CTC ATC TTC ATT CA-3′), GAPDH-rt.for (5′-TCC TGC ACC ACC AAC TGC TTA G-3′), and GAPDH-rt.rev (5′-CAC AGC CTT GGC AGC ACC AGT-3′).

PCR amplification was done using the BioRad iCycler IQ detection system and SybrGreen Super Mix (BioRad, Hercules, CA). One microgram of template cDNA was amplified with 100 ng of primers in duplicates as follows: 1 min 95°C followed by 40 cycles of 20 s at 95°C, 20 s at 55°C, and 20 s at 72°C and a final elongation cycle of 1 min at 72°C. Purity and size of product were verified by melting curve analysis and agarose gel electrophoresis, respectively. Gene expression levels were calculated using the comparative cycle threshold (C_t) method of Livak and Schmittgen (2001) with the modification of Tanaka *et al* (2003b). In brief, for each cell line the C_t value of c-Abl was subtracted from the C_t value of the respective transcript. The relative quantitation value (RQV) was obtained by calculating $2 - \Delta C_t$.

Western blot analysis

Cell lysates were obtained by lysing 1×10^6 cells in NP40 protein sample buffer (140 mM NaCl, 5 mM Mg_2Cl, 20 mM Tris-HCl pH 7.6, 1% NP40). Samples were boiled for 5 min, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide) and transferred to nitrocellulose membranes (Hybond; Amersham, Little Chalfont, UK). Membranes were probed with anti-survivin antiserum (FL-142; Santa Cruz Biotechnology, Santa Cruz, CA) and secondary horseradish peroxidase–conjugated anti-rabbit immunoglobulin G antibody (NA934; Amersham). Signals were detected by chemiluminescence.

Immunohistochemistry

Cells were cultured to subconfluence on coverslips, fixed for 20 min with 2% paraformaldehyde, quenched for 10 min with 50 mM ammonium chloride, and permeabilized for 3 min with 0.1% Triton X-100. Cells were stained with antibodies anti-hTERT (H-231; Santa Cruz), anti-survivin (FL 142; Santa Cruz), anti-VEGF-A (A20; Santa Cruz), or anti-nestin (10c2; Santa Cruz) for 60 min, followed by blocking of endogenous peroxidase activity with 1% H_2O_2 for 30 min. A biotinylated secondary antibody ($(Fab)_2$HRP sc-3837; Santa Cruz) was applied, followed by incubation with horseradish peroxidase (HRP)-avidin conjugate (BD Biosciences, Pharmingen, NJ) and diaminobenzidine (0.5 mg/ml). For immunofluorescence, the procedure was identical up to staining with primary antibodies, followed by incubation with the secondary antibody Alexa Fluor 488 (Molecular Probes, Eugene, OR) for 30 min.

Enzyme-linked immunosorbent assay

The cells were plated as described for the luciferase assays and infected 24 h later at an MOI of 0.1. Forty-eight hours post infection (p.i.) the supernatants were harvested and the amounts of mIL-2 secreted into the medium were measured by (enzyme-linked immunosorbent assay) ELISA using the mIL-2 *Ready-Set-Go!* kit (eBioscience, San Diego, USA).

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