Biochemical and Biophysical Research Communications **289,** 229–233 (2001)
doi:10.1006/bbrc.2001.5953, available online at http://www.idealibrary.com on IDEAL®

Nucleoside Analog Cytotoxicity and Bystander Cell Killing of Cancer Cells Expressing *Drosophila melanogaster* Deoxyribonucleoside Kinase in the Nucleus or Cytosol

Xinyu Zheng,*,† Magnus Johansson,* and Anna Karlsson*,[1]

**Karolinska Institute, Division of Clinical Virology, Huddinge University Hospital, S-141 86 Stockholm, Sweden; and*
†Department of Surgery, First Affiliated Hospital, China Medical University, Shenyang 110001, People's Republic of China

Received October 16, 2001

We have recently shown that the overexpression of *Drosophila melanogaster* multisubstrate deoxyribonucleoside kinase (*Dm*-dNK) in cancer cell lines increases the cells' sensitivity to several cytotoxic nucleoside analogs and the enzyme may accordingly be used as a suicide gene in combined gene/chemotherapy treatment of cancer. To further characterize the enzyme for possible use as a suicide gene, we constructed a replication-deficient retroviral vector that expressed either the wild-type enzyme that localizes to the cell nucleus or a mutant (arg247ser) that localizes to the cytosol. A thymidine kinase-deficient osteosarcoma cell line was transduced with the recombinant virus and we compared the sensitivity and bystander cell killing when the cell lines were incubated with the pyrimidine nucleoside analogs (E)-5-(2-bromovinyl)-2′-deoxyuridine and 1-β-D-arabinofuranosylthymine. In summary, we showed that the cells' sensitivity and the efficiency of bystander cell killing were not dependent on whether *Dm*-dNK was located in the nucleus or cytosol. © 2001 Academic Press

Key Words: **gene therapy; suicide gene; nucleoside kinase; nucleoside analog; nucleotide metabolism; bystander effect.**

Nucleoside kinases that phosphorylate, and thereby pharmacologically activate, cytotoxic nucleoside analogs are studied for their possible use in suicide gene therapy of cancer (1). The most carefully studied suicide gene is the herpes simplex virus type-1 thymidine kinase (HSV-1 TK) used in combination with the guanosine nucleoside analog ganciclovir (2–5). Cells expressing HSV-1 TK phosphorylate ganciclovir to the triphosphate form that will be incorporated into nuclear DNA during DNA replication and repair. Incorporation of ganciclovir into DNA causes termination of DNA chain elongation and results in cell death (6). Phosphorylated ganciclovir may be transported between cells via gap-junctions, and cells located adjacent to the cells expressing HSV-1 TK are also killed (7–9). The killing of adjacent cells, known as the "bystander effect," is an important factor for the success of suicide gene therapy since currently available gene delivery systems does not allow introduction of the suicide gene in all tumor cells.

In attempts to improve suicide gene therapy, combinations of suicide nucleoside kinases and nucleoside analogs other than HSV-1 TK and ganciclovir are being studied. Alternative nucleoside kinases include thymidine kinases from different members of the herpes virus family as well as human nucleoside kinases (10–12). Genetic engineering of nucleoside kinases is also used in order to create nucleoside kinases with improved kinetic properties for the phosphorylation of nucleoside analogs (13, 14). In summary, these studies suggest that the kinetic properties of the nucleoside kinase are important determinants for the efficiency of suicide gene therapy.

We have recently studied a deoxyribonucleoside kinase from the fruit fly *Drosophila melanogaster* (*Dm*-dNK) for possible use as a suicide gene (15, 16). *Dm*-dNK has broad substrate specificity with regard to both purine and pyrimidine nucleoside analog phosphorylation and exhibit a higher catalytic rate than previously studied nucleoside kinases (17, 18). These properties suggest that the enzyme may be an efficient suicide gene. *Dm*-dNK retains its activity when expressed in human cells, localizes to the cell nucleus, and increases the cells' sensitivity to several cytotoxic nucleoside analogs (15). In the present study, we have further characterized the use of *Dm*-dNK as a suicide gene. We created a replication deficient retrovirus vector that expressed *Dm*-dNK fused to the green fluorescent protein (GFP) to facilitate detection of the expressed enzyme in living cells. We also created a

[1] To whom correspondence and reprint requests should be addressed. Fax: +46-8-58587933. E-mail: anna.karlsson@mbb.ki.se.

0006-291X/01 $35.00
Copyright © 2001 by Academic Press
All rights of reproduction in any form reserved.

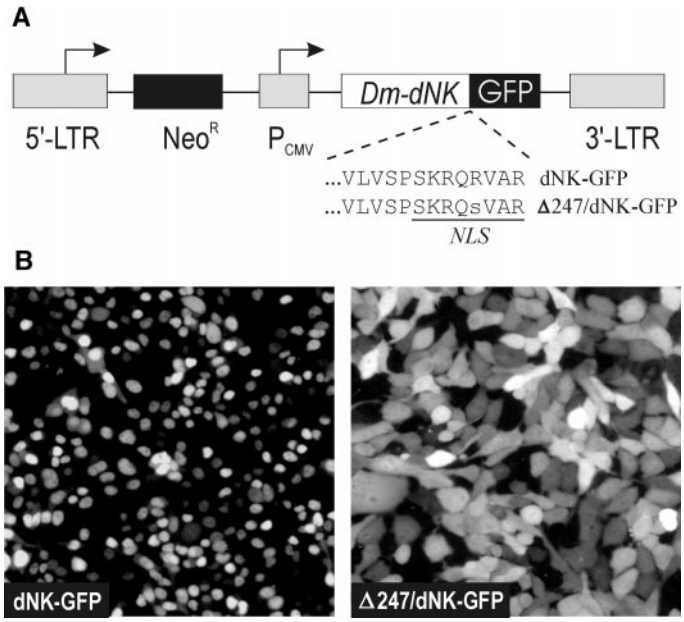

FIG. 1. Expression of *Dm*-dNK in the nucleus or cytosol of osteosarcoma cells. (A) Retroviral vector constructs used to express wild-type nuclear *Dm*-dNK (dNK–GFP) and the cytosolic mutant *Dm*-dNK (Δ247/dNK–GFP) in fusion with GFP. (B) GFP fluorescence microscopy of osteosarcoma cells transduced with the retroviral vectors. NLS, nuclear localization signal; P_{CMV}, cytomegalovirus promoter; LTR, long terminal repeats; Neo^R, neomycin resistance gene.

mutant cytosolic *Dm*-dNK and compared the cytotoxicity and bystander effects for the nucleoside analogs (E)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) and 1-β-D-arabinofuranosylthymine (araT) when the enzyme was expressed in either the nucleus or cytosol. In summary, we showed that the subcellular location of *Dm*-dNK expression did not affect the cells' sensitivity to the nucleoside analogs or the efficiency of bystander cell killing.

MATERIALS AND METHODS

Cell culture. RetroPack PT67 packaging cells (CLONTECH) and TK-deficient osteosarcoma cells (a gift from Professor J. Balzarini, Rega Institute, Leuven, Belgium) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum (Gibco BRL), 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were grown at 37°C in a humidified incubator with a gas phase of 5% CO_2.

Construction of retrovirus vectors and transduction of cancer cells. We used the pLEGFP-N1 retroviral vector (CLONTECH) to express the *Dm*-dNK cDNA in fusion with the green fluorescent protein (dNK–GFP) (Fig. 1). The wild-type *Dm*-dNK (wt*Dm*-dNK) and the mutant *Dm*-dNK (Δ247/*Dm*-dNK) were constructed as described (15) and cloned into the *Xho*I–*Bam*HI site of the pLEGFP-N1 vector. The plasmid was purified using the NucleoBond plasmid purification kit (CLONTECH). The DNA sequence of the constructed plasmid was verified by DNA sequence determination using an ABI310 automated DNA sequencer (Perkin–Elmer).

The constructed pLEGFP-N1 plasmid vectors were transfected into the PT67 packaging cells using LipofectAmine (Life Technology Inc.) according to the protocol provided by the supplier. The medium from the transfected cells was collected 48 h after transfection, filtered through a 0.45-μm filter, and diluted twofold with fresh medium. The osteosarcoma cells were incubated with the virus-containing medium for 48 h and then cultured continuously for 3 weeks in the presence of 1.0 mg/ml Geneticin (Gibco BRL).

Western blot analysis and enzyme assays. Protein extracts were prepared as described (15). The protein extracts were separated by 1.2% SDS/PAGE gel electrophoresis and electrotransferred to the nitrocellulose membrane. The membrane blots were probed for 1 h with a polyclonal anti-GFP antibody alkaline phosphatase conjugate (Clontech). The alkaline phosphatase was visualized by incubation with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma). The enzyme assays were performed as described (15). 3.0 μM [*methyl*-^{3}H]dThd (Moravek Biochem) and 2 μM unlabeled dThd (Sigma) were used in the reaction.

Autoradiography. The cells were cultured on poly-L-lysine-coated chamber slides (Nunc, Inc.) for 24 h and labeled with [*methyl*-^{3}H]dThd (Moravek Biochem) for 12 h. The slides were rinsed twice with PBS, fixed for 10 min in methanol:acetic acid (3:1), and washed three times with ice-cold 10% TCA, once with water, and once with methanol. The slides were coated with Hypercoat photoemulsion (Amersham) and exposed 1–3 weeks at 4°C. The autoradiograms were developed using D-11 developer (Kodak).

Cell proliferation and bystander killing assays. 1-β-D-arabinofuranosylthymine (araT) was obtained from Lilly Research Laboratories. (E)-5-(2-Bromovinyl)-2′-deoxyuridine (BVDU) was a gift from Professor J. Balzarini (Rega Institute, Leuven, Belgium). The cells were plated at ≈2000 cells/well in 96-well plates. Nucleoside analogs were added after 24 h and the medium containing the nucleoside analogs was changed once during the 4-days incubation. Cell survival was assayed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Boehringer Mannheim) after 4 days of drug exposure. Each experiment was performed in triplicate.

The assay for bystander cell killing was performed as described (16). Tumor cells expressing *Dm*-dNK were mixed at different ratios with their respective parental cell lines. To promote cell contacts, the mixed cells were plated in 24-well plates at 3×10^5 cells/well. The following day, confluent cells were treated with BVDU. After 24 h incubation, cells were trypsinized and a 1:100 dilution of the cells was distributed into 96-well plates in five replicates. Cells were cultured subsequently in the presence of BVDU for 2–3 days, until cells without BVDU reached confluence. Cell survival was determined as described above.

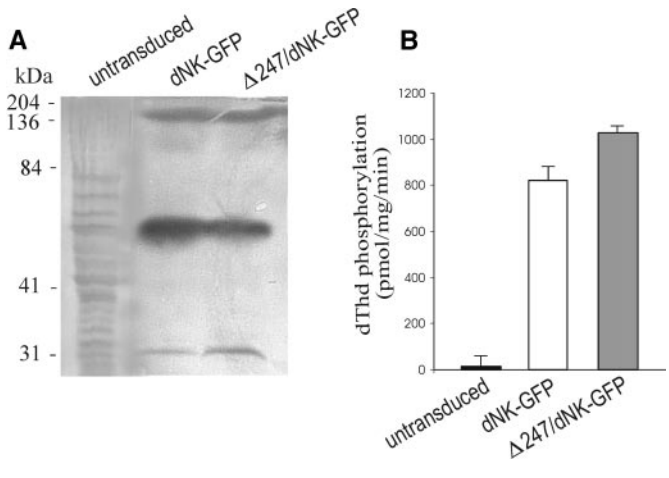

FIG. 2. Expression and activity of dNK–GFP in the transduced cells (A) Western blot analysis of cell protein extracts using anti-GFP antibodies. (B) *Dm*-dNK activity, determined as dThd phosphorylation, in crude extracts of the untransduced and transduced osteosarcoma cells.

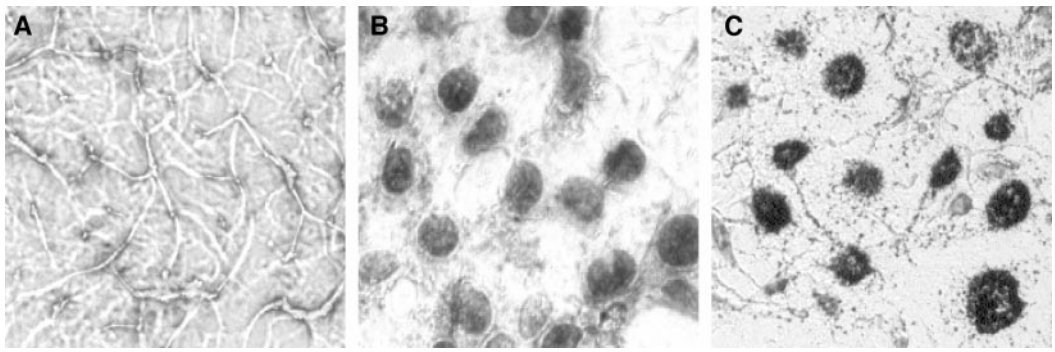

FIG. 3. [^{3}H]dThd autoradiography of the TK-deficient untransduced osteosarcoma cells (A), expressing nuclear dNK–GFP (B) or cytosolic (Δ247/dNK–GFP).

RESULTS

Expression of Dm-dNK in the Nucleus or Cytosol in a Cancer Cell Line

We decided to express *Dm*-dNK in the nucleus or in the cytosol in a cancer cell line to compare the cells' sensitivity to nucleoside analogs and the efficiency of bystander cell killing when the enzyme was located in either of the two subcellular compartments. Expression of wild-type *Dm*-dNK in cell lines results in nuclear localization of the enzyme due to a nuclear localization signal in the C-terminal region of the protein (15). Site-directed mutagenesis of arginine-247 to serine abolishes nuclear import of the protein resulting in a predominant cytosolic localization of the enzyme (15). We constructed replication deficient retroviral vectors to express either the wild-type nuclear *Dm*-dNK (dNK–GFP) or the cytosolic arginine-247 *Dm*-dNK mutant (Δ247/dNK–GFP) (Fig. 1). The proteins were expressed in fusion with the green fluorescent protein (GFP) in order to easily visualize the subcellular location of the recombinant enzymes *in vivo*.

We transduced a thymidine kinase-1-deficient osteosarcoma cell line with the recombinant retrovirus vector. After selection for stably transfected cells, >90% of the cells exhibited green fluorescence (Fig. 1). The cells transduced with the vector encoding dNK–GFP exhibited fluorescence in the nucleus whereas the cells transduced with the vector encoding Δ247/dNK–GFP showed fluorescence predominantly in the cytosol. Western blot analysis with anti-GFP antibodies detected similar levels of the ≈60-kDa fusion proteins in the cells expressing either the nuclear dNK–GFP or the cytosolic Δ247/dNK–GFP (Fig. 2).

To test the enzymatic activity of the *Dm*-dNK–GFP fusion proteins, we assayed dThd phosphorylation activity in cell protein extracts (Fig. 2B). The dThd kinase activity was increased ≈60-fold in the cells expressing either the nuclear dNK–GFP or the cytosolic Δ247/dNK–GFP compared to the untransduced parent cell line. There was no significant difference in *Dm*-dNK activity between the cells expressing dNK–GFP in the cytosol and in the nucleus. We used autoradiography to *in situ* visualize incorporation of [^{3}H]dThd into DNA (Fig. 3). Cells expressing nuclear dNK–GFP or cytosolic Δ247/dNK–GFP showed dark staining of cell nuclei indicating incorporation of [^{3}H]dThd into nuclear DNA, whereas no incorporation of [^{3}H]dThd into nuclear DNA was detected in the thymidine kinase-deficient parent cell line.

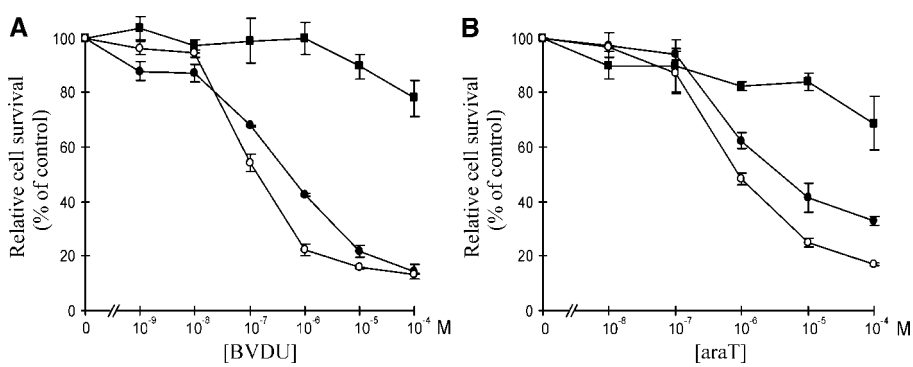

FIG. 4. Sensitivity of the untransduced osteosarcoma cells (■) and the cells expressing dNK–GFP (○) or Δ247/dNK–GFP (●) to BVDU and araT.

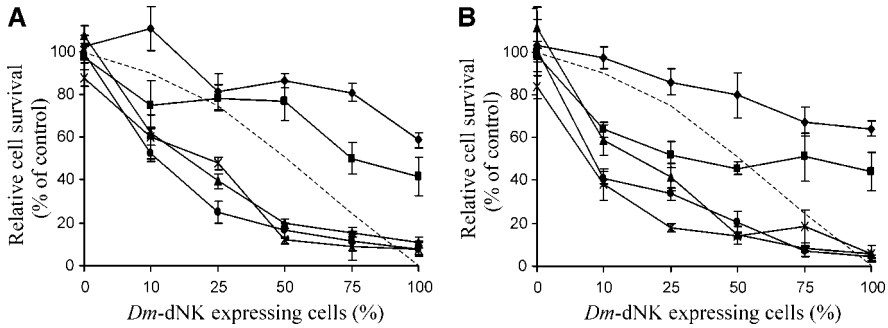

FIG. 5. *In vitro* bystander cell killing of untransduced osteosarcoma cells mixed in different ratios with dNK–GFP (A) or Δ247/dNK–GFP (B). The cells were incubated in the presence of 0.01 μM (◆), 0.1 μM (■), 1 μM (▲), 10 μM (●), or 100 μM (X) BVDU. The cell survival (mean of five experiments ± standard deviation) is expressed relative to cells incubated without BVDU. The dashed line indicates the calculated cell survival if only *Dm*-dNK expressing cells had been killed and no bystander effect occurred.

Nucleoside Analog Sensitivity and Bystander Cell Killing

We determined the sensitivity of the transduced cells to the pyrimidine nucleoside analogs BVDU and araT (Fig. 4). The two *Dm*-dNK–GFP expressing osteosarcoma cell lines were more sensitive to the nucleoside analogs than the untransduced cells. The cells expressing *Dm*-dNK in the nucleus or in the cytosol exhibited ≈50- to 500-fold lower IC_{50} for the investigated compounds compared with the untransduced cells. There were no differences in nucleoside analog sensitivity between the cells expressing *Dm*-dNK in the nucleus and the cytosol.

The cells expressing dNK–GFP or Δ247/dNK–GFP were mixed at different ratios with untransduced cells. BVDU was added to the mixed cells at concentrations from 0.01 to 100 μM and incubated for 4 days. A bystander effect was found both in the dNK–GFP or Δ247/dNK–GFP expressing osteosarcoma cells (Fig. 5). There were no differences in bystander killing between the cells expressing *Dm*-dNK in the nucleus and in the cytosol after incubation with BVDU.

DISCUSSION

We have expressed *Dm*-dNK fused to GFP in the nucleus or in the cytosol of a cancer cell line and shown that the cellular sensitivity to cytotoxic nucleoside analogs and the bystander cell killing is not dependent on the subcellular location of the enzyme. Molecules with low molecular mass, such as phosphorylated nucleoside analogs, are probably crossing the nuclear membrane by free diffusion through the nuclear pore complexes. It has been shown that the intracellular location does not affect the cytotoxicity for other nucleoside kinases such as herpes simplex virus type-1 thymidine kinase and deoxycytidine kinase (19–21). Interestingly, the bystander cell killing was also similar when the nucleoside analogs were phosphorylated in the nucleus or in the cytosol. Bystander cell killing is mediated by the intercellular transport of phosphorylated nucleoside analogs via gap junctions in the cell membrane. When designing the experiments for the study, we hypothesized that the bystander cell killing might be more efficient when the nucleoside analogs were phosphorylated in the cytosol due to the proximity to the adjacent cells. However, our findings rather suggest that the nuclear and cytosolic dNTP pools, as well as the phosphorylated nucleoside analog pools in these compartments, are rapidly equilibrated.

Our findings also show that *Dm*-dNK retains enzymatic activity when fused to GFP, and the use of such fusion protein will facilitate detection of the expressed suicide gene in cells and tissues. However, the enzyme activity determined as dThd phosphorylation in crude cell extracts was 7-fold lower compared to a previous study when *Dm*-dNK was expressed without the GFP fusion tag (15). The lower level of total dThd activity is also associated with a decreased sensitivity to BVDU and araT. It is possible that fusion to GFP decreases the expression of the enzyme, but the C-terminal fusion to GFP may also affect the catalytic properties of *Dm*-dNK. Modification of the C-terminal region of *Dm*-dNK has been shown to affect the kinetic properties of the enzyme (22, 23). It is possible that fusing GFP to the N-terminus of *Dm*-dNK may be preferred when a GFP fusion protein is required for easy visualization of *Dm*-dNK expression.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Medical Research Council, the Swedish Cancer Foundation, the Swedish Foundation of Strategic Research, the European Commission, and the Petrus and Augusta Hedlunds Foundation.

REFERENCES

1. Springer, C. J., and Niculescu-Duvaz, I. (2000) Prodrug-activating systems in suicide gene therapy. *J. Clin. Invest.* **105,** 1161–1167.

2. Balzarini, J., de Clercq, E., Ayusawa, D., and Seno, T. (1985) Murine mammary FM3A carcinoma cells transformed with the herpes simplex virus type 1 thymidine kinase gene are highly sensitive to the growth-inhibitory properties of (E)-5-(2-bromovinyl)-2′-deoxyuridine and related compounds. *FEBS Lett.* **185,** 95–100.
3. Moolten, F. L. (1986) Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: Paradigm for a prospective cancer control strategy. *Cancer Res.* **46,** 5276–5281.
4. Moolten, F. L., and Wells, J. M. (1990) Curability of tumors bearing herpes thymidine kinase genes transferred by retroviral vectors. *J. Natl. Cancer Inst.* **82,** 297–300.
5. Culver, K. W., Ram, Z., Wallbridge, S., Ishii, H., Oldfield, E. H., and Blaese, R. M. (1992) *In vivo* gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. *Science* **256,** 1550–1552.
6. Reardon, J. E. (1989) Herpes simplex virus type 1 and human DNA polymerase interactions with 2′-deoxyguanosine 5′-triphosphate analogues. Kinetics of incorporation into DNA and induction of inhibition. *J. Biol. Chem.* **264,** 19039–19044.
7. Freeman, S. M., Abboud, C. N., Whartenby, K. A., Packman, C. H., Koeplin, D. S., Moolten, F. L., and Abraham, G. N. (1993) The "bystander effect": Tumor regression when a fraction of the tumor mass is genetically modified. *Cancer Res.* **53,** 5274–5283.
8. Mesnil, M., Piccoli, C., Tiraby, G., Willecke, K., and Yamasaki, H. (1996) Bystander killing of cancer cells by herpes simplex virus thymidine kinase gene is mediated by connexins. *Proc. Natl. Acad. Sci. USA* **93,** 1831–1835.
9. Rubsam, L. Z., Boucher, P. D., Murphy, P. J., KuKuruga, M., and Shewach, D. S. (1999) Cytotoxicity and accumulation of ganciclovir triphosphate in bystander cells cocultured with herpes simplex virus type 1 thymidine kinase-expressing human glioblastoma cells. *Cancer Res.* **59,** 669–675.
10. Degreve, B., Andrei, G., Izquierdo, M., Piette, J., Morin, K., Knaus, E. E., Wiebe, L. I., Basrah, I., Walker, R. T., De Clercq, E., and Balzarini, J. (1997) Varicella-zoster virus thymidine kinase gene and antiherpetic pyrimidine nucleoside analogues in a combined gene/chemotherapy treatment for cancer. *Gene Ther.* **4,** 1107–1114.
11. Manome, Y., Wen, P. Y., Dong, Y., Tanaka, T., Mitchell, B. S., Kufe, D. W., and Fine, H. A. (1996) Viral vector transduction of the human deoxycytidine kinase cDNA sensitizes glioma cells to the cytotoxic effects of cytosine arabinoside *in vitro* and *in vivo*. *Nat. Med.* **2,** 567–573.
12. Zhu, C., Johansson, M., Permert, J., and Karlsson, A. (1998) Enhanced cytotoxicity of nucleoside analogs by overexpression of mitochondrial deoxyguanosine kinase in cancer cell lines. *J. Biol. Chem.* **273,** 14707–14711.
13. Kokoris, M. S., Sabo, P., Adman, E. T., and Black, M. E. (1999) Enhancement of tumor ablation by a selected HSV-1 thymidine kinase mutant. *Gene Ther.* **6,** 1415–1426.
14. Christians, F. C., Scapozza, L., Crameri, A., Folkers, G., and Stemmer, W. P. (1999) Directed evolution of thymidine kinase for AZT phosphorylation using DNA family shuffling. *Nat. Biotechnol.* **17,** 259–264.
15. Zheng, X., Johansson, M., and Karlsson, A. (2000) Retroviral transduction of cancer cell lines with the gene encoding *Drosophila melanogaster* multisubstrate deoxyribonucleoside kinase. *J. Biol. Chem.* **275,** 39125–39129.
16. Zheng, X., Johansson, M., and Karlsson, A. (2001) Bystander effects of cancer cell lines transduced with the multisubstrate deoxyribonucleoside kinase of *Drosophila melanogaster* and synergistic enhancement by hydroxyurea. *Mol. Pharmacol.* **60,** 262–266.
17. Munch-Petersen, B., Piskur, J., and Sondergaard, L. (1998) Four deoxynucleoside kinase activities from *Drosophila melanogaster* are contained within a single monomeric enzyme, a new multifunctional deoxynucleoside kinase. *J. Biol. Chem.* **273,** 3926–3931.
18. Johansson, M., van Rompay, A. R., Degreve, B., Balzarini, J., and Karlsson, A. (1999) Cloning and characterization of the multisubstrate deoxyribonucleoside kinase of *Drosophila melanogaster*. *J. Biol. Chem.* **274,** 23814–23819.
19. Johansson, M., Brismar, S., and Karlsson, A. (1997) Human deoxycytidine kinase is located in the cell nucleus. *Proc. Natl. Acad. Sci. USA* **94,** 11941–11945.
20. Degreve, B., Johansson, M., De Clercq, E., Karlsson, A., and Balzarini, J. (1998) Differential intracellular compartmentalization of herpetic thymidine kinases (TKs) in TK gene-transfected tumor cells: Molecular characterization of the nuclear localization signal of herpes simplex virus type 1 TK. *J. Virol.* **72,** 9535–9543.
21. Zhu, C., Johansson, M., and Karlsson, A. (2000) Incorporation of nucleoside analogs into nuclear or mitochondrial DNA is determined by the intracellular phosphorylation site. *J. Biol. Chem.* **275,** 26727–26731.
22. Munch-Petersen, B., Knecht, W., Lenz, C., Sondergaard, L., and Piskur, J. (2000) Functional expression of a multisubstrate deoxyribonucleoside kinase from *Drosophila melanogaster* and its C-terminal deletion mutants. *J. Biol. Chem.* **275,** 6673–6679.
23. Knecht, W., Munch-Petersen, B., and Piskur, J. (2000) Identification of residues involved in the specificity and regulation of the highly efficient multisubstrate deoxyribonucleoside kinase from *Drosophila melanogaster*. *J. Mol. Biol.* **301,** 827–837.