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Green Fluorescent Protein (GFP) as a Marker for Cell Viability After UV Irradiation

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Generation of Chinese Hamster Ovary (CHO) cell lines stably expressing green fluorescent protein (GFP) was achieved using a plasmid vector that encoded the red-shifted pCX-xGFP under the control of a strong hybrid promoter composed of a CMV enhancer and a β-actin/β-globin gene promoter. Cotransfection of the promoter-less pSV2-Neo helper plasmid transmitting neomycin resistance was followed by selection with the antibiotic G418. Constitutive GFP expression could be visualized in living and fixed cells using fluorescence spectroscopy, fluorescence microscopy, and flow cytometry. DNA repair-proficient (AA8) and deficient (UV5) CHO strains were used for survival tests after UVC irradiation. Cells carrying the GFP construct (AA8-pGFP, UV5-pGFP) show the same response to UV irradiation (colony forming ability) as their nontransformed parental cell lines (AA8, UV5). Using GFP as a marker for cell viability, cells were harvested after certain postirradiation growth periods and the numbers of GFP expressing cells and fluorescence intensities were determined by FACS analysis. Generally, GFP fluorescence in irradiated cells is not seen when cell membranes are damaged (leak-out of the soluble GFP). Irradiated cells without membrane damage express GFP continuously (leading to a dose-dependent increase in GFP contents).

KEY WORDS: Green fluorescent protein (GFP); constitutive GFP expression; visualization of GFP fluorescence; UV exposure of mammalian cells; Chinese Hamster Ovary (CHO) cell lines of different repair capabilities.

INTRODUCTION

The introduction of reporter genes into cultured mammalian cells represents an important means of investigating cellular radiosensitivity. In studies of this kind, the gene or promoter element of interest is usually inserted into a vector, where the expression of the reporter gene either is driven by a suitable selected heterologous promoter and other elements or is a consequence of the cellular response to radiation.

Expression of green fluorescent protein (GFP), originally isolated from the bioluminescent jellyfish *Aequorea victoria*, represents a unique method for fluorescent labeling of viable cells, with many potential applications [3]. GFP is a 27-kD monomer consisting of 238 amino acids. The purified protein retains its fluorescence proficiency under many severe conditions including heat (T_m = 70°C), pH extremes (pH 7–12), exposure to urea (8 *M*), and incubation with low concentrations of detergents (1% SDS) [3]. Expression of GFP is superior to other reporter assays, as GFP fluorescence requires only exposure to UV or blue light, unlike other bioluminiscent reporter systems, which require incubation of the cells with specific substrates or cofactors to emit light. GFP fluorescence can thus be monitored in living cells.

UV irradiation is known to induce certain classes of genes [5,8,12] and to decrease the expression of other classes of genes [2]. This modulation of gene expression is thought to be mediated to some extent by promoter

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specific interactions and regulatory elements like transcription factors [7,11]. We, therefore, are interested in the question whether or not GFP expression is a suitable reporter for radiation-induced gene activation. Of special importance in this respect is the stability of the GFP molecule itself and of vector-driven GFP expression after irradiation.

MATERIALS AND METHODS

Chinese Hamster Ovary (CHO) Cells

Cell Strains

The AA8 Chinese hamster ovary cell line (ATCC CRL-1859) is the repair-proficient parental clone of many repair-deficient mutants such as the UV-sensitive mutant cell line UV5 (ATCC CRL-1865), which is detective in nucleotide excision repair [10]. The UV5 cell line is sensitive to UV exposure, and to bulky-adduct mutagens, belongs to the excision repair complementation group 2, and is defective in the incision step of the repair of UV-induced base damage in DNA. The defective gene product (ERCC2) of the hamster cell line is functionally and structurally homologous to the human XPD variant of *Xeroderma pigmentosum.*

The AA8-pGFP and UV5-pGFP cell lines were established as described below.

Routine Cultivation

The CHO cell lines AA8 and UV5 grow under standard conditions (DMEM, 5% FCS, 37°C, 5% CO_2/95% air) as attached cells, with a doubling time of about 12 and 13 h, respectively. Cultures were split (1:40) and passaged every 5–7 days using standard trypsinization procedures.

Transfection Conditions and Selection of Stable Clones

The pCX-xGFP vector contains a strong hybrid promoter composed of a CMV enhancer and a β-actin/β-globin gene promoter upstream of the GFP gene [1]. Purified pCX-xGFP and pSV2-Neo (Clontech) DNAs were prepared using standard protocols.

The AA8 and UV5 cell lines were transfected using Tfx-50 Reagent (Promega), with some minor modifications of the manufacturer's protocol. Immediately before transfection 1 μg of the promoter-less helper plasmid pSV2-Neo, containing the gene for neomycin resistance, and 6 μg pCX-xGFP DNA were mixed with 21 μl of liposomes and added to 1.4 ml of serum-containing DMEM. Semiconfluent cells were incubated in six-well plates at 37°C and 5% CO_2 in the presence of the DNA–liposome complex (200 μl) for 2 h, after which the solution was diluted by the addition of 4 ml standard growth medium.

At 24–48 h after transfection the cells were split 1:25 and the aminoglycoside antibiotic G418 (Calbiochem–Novabiochem), a neomycin analogue, was added to the medium (1.5 mg/ml). After several rounds of cell divisions (usually 1 to 2 weeks, with regular exchange of G418 containing medium), only cells with stably integrated constructs survived the selection conditions. GFP expressing clones were obtained by picking single stably transfected colonies and serial dilution into petri dishes. GFP expressing clones were maintained in medium containing 1.5 mg/ml G418.

UVC Exposure

UVC irradiation was performed using a germicidal lamp (NN8/15; Heraeus); UV dosimetry was performed using a calibrated UVX-radiometer (UVP Inc.). For exposure with UVC, medium was sucked off from the petri dishes and the cells were rinsed twice with phosphate-buffered saline (PBS). Immediately after UV exposure, cells were incubated under standard conditions (DMEM, 5% FCS, 37°C, 5°CO_2/95% air).

Measurement of Colony Formation for Cellular Survival

Cellular UV sensitivity of cultured CHO cells was tested using the colony forming ability test. For survival experiments cells from confluent layers were plated 16–20 h before UV exposure at appropriate cell densities. The cell numbers per petri dish were adjusted to compensate for plating efficiency of the cell line and any anticipated lethal effect of the treatment, in order to obtain between 20 and 60 colonies per culture dish.

UVC exposure was performed as already described. After UV exposure cells were allowed to grow for 14–18 days without any change of medium, colonies were stained with crystal violet (1 mg/ml in 4% paraformaldehyde solution). Only colonies containing more than 50 cells were scored as survivors. Experiments were performed with six petri dishes per dose and repeated at least twice. All experimental data were standardized to $S_D/S_{D=o}$, where S_D and $S_{D=o}$ were the surviving fractions of treated and untreated cells, respectively. Results are given as survival curves according to Eq. (1), where D

is the dose (Jm^{-2}), S is the surviving fraction per dose, and D_0 and n are dose-proportional constants:

$$S = 1 - (1 - e^{-D/D_0})^n \quad (1)$$

Detection of GFP Expression

GFP expressing cells were analyzed either in vital form or after different fixation procedures [4% paraformaldehyde in PBS, 70% ice-cold ethanol, 100% ice-cold methanol, methanol/acetic acid (3:1, v:v)] and counterstaining with DNA-specific dyes (DAPI, $2 \cdot 10^{-6}$ M; Hoechst 33258, $1.25 \cdot 10^{-6}$ M; propidium iodide, 5 μg/ml). Staining of DNA using propidium iodide (PI) requires simultaneous digestion of double-stranded RNA with RNase A (100 U/ml, 30 min).

Fluorescence Microscopy and Spectroscopy

For fluorescence microscopic analysis GFP expressing cell clones AA8-pGFP and UV5-pGFP were inoculated on 35-mm coverslips and examined using a Zeiss Axiovert 135 inverted microscope equipped with a filter set suitable for fluorescein detection (Filter Set 9; excitation, BP 450–490 nm; emission, LP 520 nm), DAPI detection (Filter Set 2; excitation, G 365 nm; emission, LP 420 nm), and PI detection (Filter Set 14; excitation, LP 510 nm and KP 560 nm; emission, LP 590 nm). Photographs were taken with a 20× and a 32× objective. For fluorescence spectroscopy cells were grown in 80-cm^2 flasks and harvested by trypsinization. Fluorescence spectra were recorded using an Hitachi fluorimeter.

Flow Cytometry

The AA8-pGFP and UV5-pGFP cell lines were harvested and analyzed on a FACScan flow cytometer (Becton–Dickinson) equipped for fluorescein isothiocyanate and propidium iodide detection at an excitation wavelength of 488 nm.

RESULTS

Visualization of GFP Expression

Like several other GFPs CX-xGFP has a strong red-shifted excitation peak at 488 nm and an emission maximum at 509-nm wavelength (Fig. 1). Thus GFP-expressing cells can be visualized using fluorescence microscopy, spectroscopy, and FACS analysis. Cells transfected with the pCX-xGFP construct display diffuse fluorescence throughout the cytoplasm and nucleus (Fig. 2). These data show that GFP is expressed well in CHO cells, as has been already demonstrated for a number of cell lines and different applications. After fixation with different common fixatives, GFP fluorescence is not stable. Only in the case of paraformaldehyde fixation (Fig. 3) is the fluorescence yield comparable to living cells. Fixation with ethanol or methanol destroys the GFP fluorescence nearly completely. Staining of DNA using highly specific dyes such as DAPI and Hoechst 33258 is not suitable for FACS analysis. Staining with propidium iodide, the common dye for FACS applications, requires digestion of RNA for proper DNA quantification. In RNase-digested preparations (Fig. 4), only about 40–60% of the AA8-pGFP cells display GFP fluorescence. FACS analysis of propidium iodide-stained DNA (Fig. 3) of ethanol-fixed cell samples reveals the typical cell cycle distribution for exponentially growing cells. Fixation with paraformaldehyde followed by PI counterstaining was not found to produce reliable DNA measurements by FACS analysis, whereas for fluorescence microscopy the quality is satisfactory.

Cellular Survival After X-Irradiation

Survival curves (Fig. 5, Table I) of the wild-type strains AA8 and AA8-pGFP obtained after UVC exposure are of curvilinear shape, with D_0 in the same range. Dose–effect curves of the repair-deficient mutants UV5 and

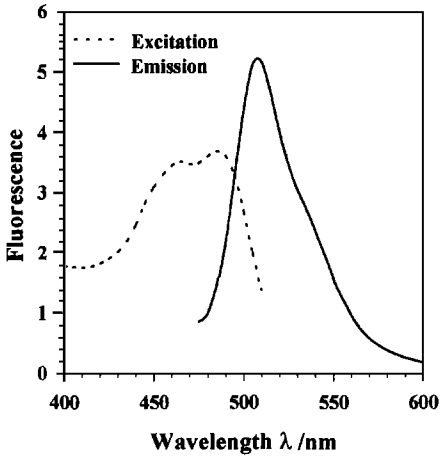

Fig. 1. Excitation and emission spectra of GFP expressing living cells (CHO AA8). The excitation data were obtained with emission at 540 nm and the emission data were obtained with excitation at 450 nm.

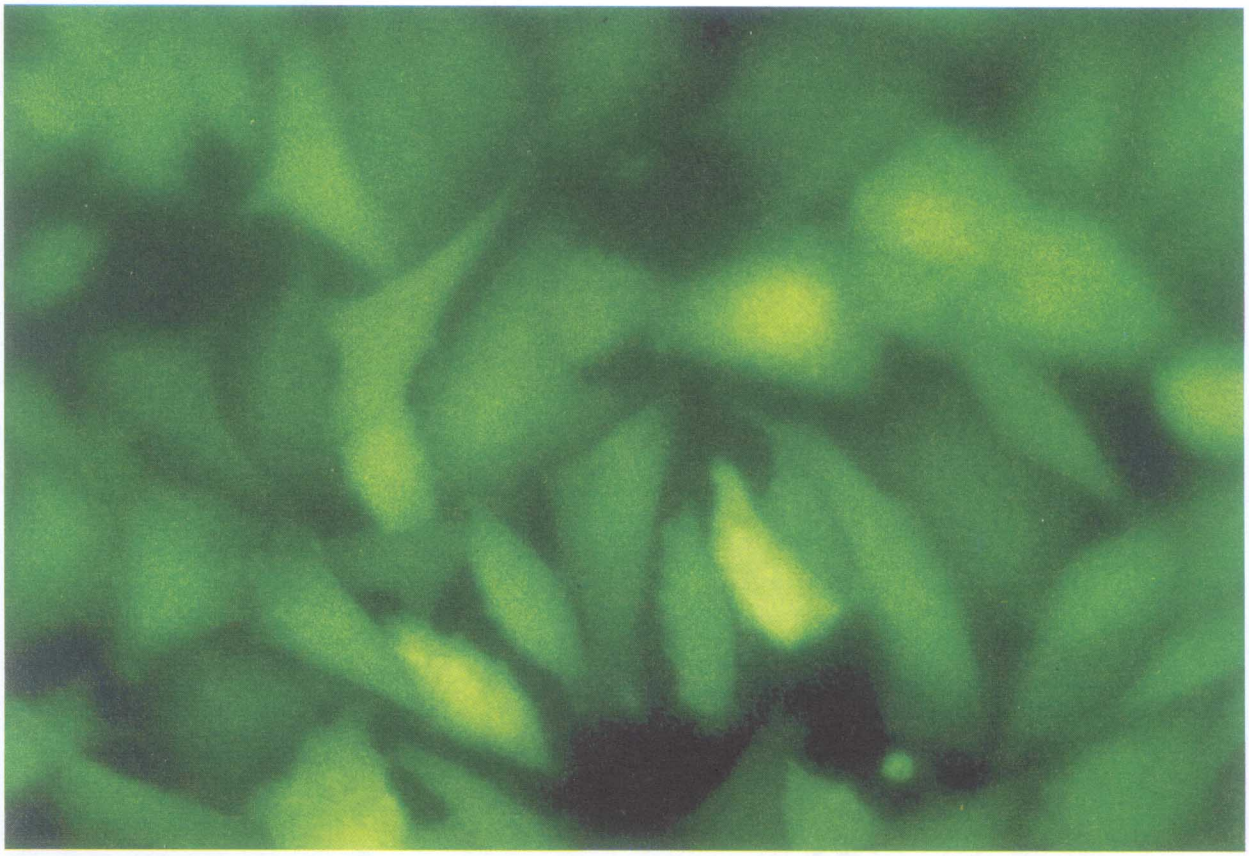

Fig. 2. Monolayer culture of the stably transfected CHO AA8-pGFP cell clone I. Life microscopy was performed using a Zeiss Axiovert 135 inverted microscope with a filter set suitable for fluorescein detection (Filter Set 9; excitation, BP 450–490 nm; emission, LP 520 nm). The high background fluorescence is due to autofluorescence of the culture flask.

UV5-pGFP are purely exponential. Cells carrying the GFP construct show no difference in cellular survival as measured by the CFA test, neither in wild-type nor in repair-proficient clones. Colonies, of both the stably transfected wild-type and mutant clones, were inspected for GFP expression by fluorescence microscopic surveillance. In any case, surviving cells produced green colonies (results not shown). Sectoring within single colonies could not be observed.

GFP Expression After UVC Exposure

For using GFP as a marker for cell viability UVC-irradiated cells were harvested after certain postirradiation growth periods and the numbers of GFP expressing cells and fluorescence intensities were determined by FACS analysis using formaldehyde-fixed cells (Fig. 6). In nonirradiated populations, nearly all cells were found to be positive for GFP expression. In short-term culture (1–4 h) the same results were obtained for nonirradiated cells and cells grown after UV-exposure. For long-term cultures (>4 h), in UVC-exposed populations, two subpopulations of cells could be identified: (i) GFP-negative (GFP content, 10^0 to $3 \cdot 10^1$) and (ii) GFP-positive (GFP content, $>3 \cdot 10^1$) cells. With increasing fluences and increasing incubation periods after UVC exposure, the numbers of GFP-negative cells increase as the numbers of GFP-positive cells decrease. The distributions for GFP-positive cells are shifted to higher GFP concentrations with increasing fluences. Seventy-two hours after UVC exposure, about 40% of cells are GFP-negative for AA8-pGFP exposed to 25 Jm^{-2} and for UV5-pGFP exposed to 7.5 Jm^{-2}.

DISCUSSION

GFP is a versatile tool for studying gene expression in mammalian cells. A number of GFP variants has been developed which differ significantly in fluorescence prop-

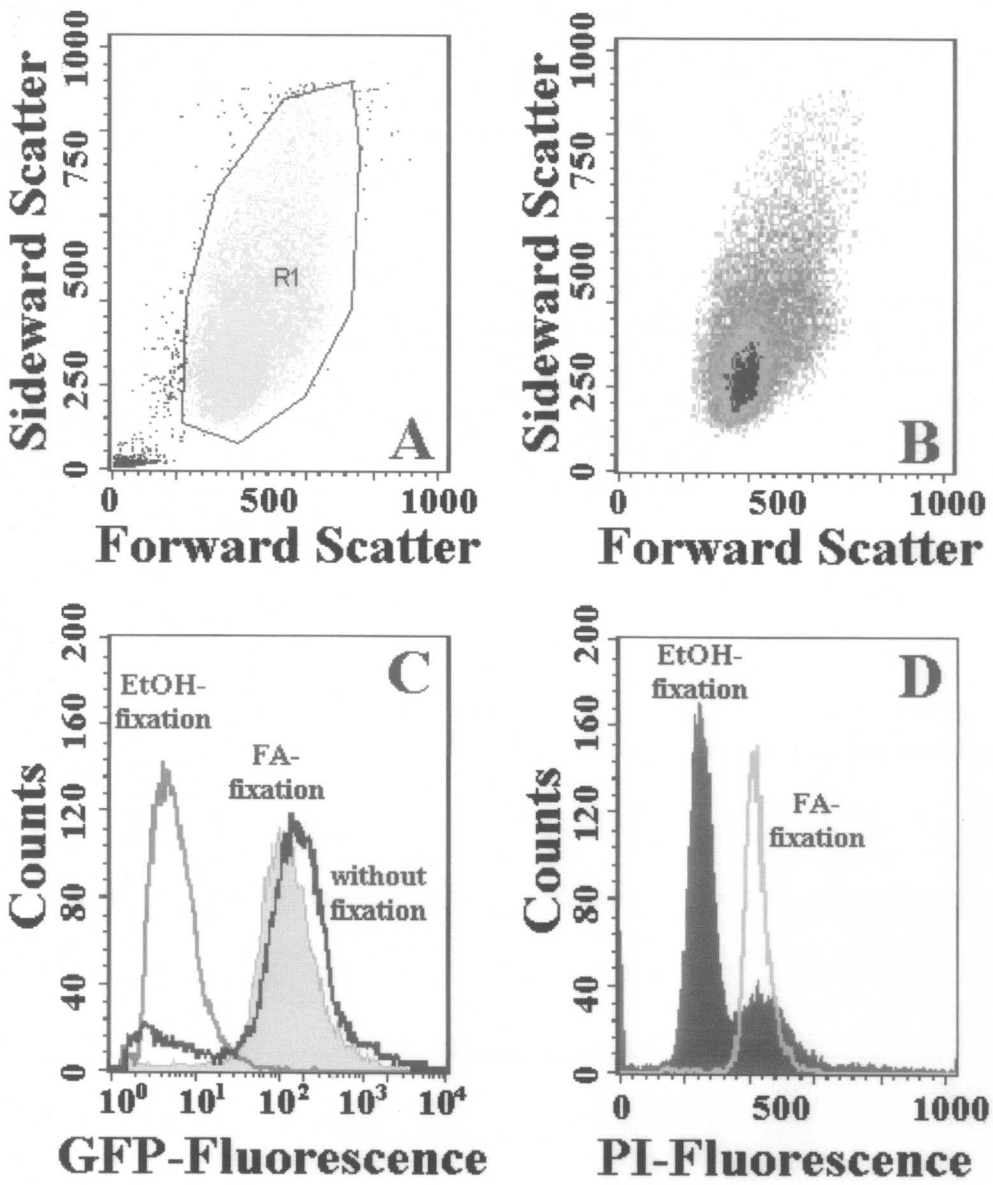

Fig. 3. FACS analysis of GFP expressing cells after fixation of AA8-pGFP cells using 4% paraformaldehyde or 70% ethanol compared to living cells: (A) dot plot showing the scatter distribution of the cell population without fixation and the window of gated cells; (B) frequency distribution of the gated cells shown in A; (C) the histogram plot for GFP expressing living and fixed cells, showing that GFP fluorescence could be maintained in paraformaldehyde-fixed cells, whereas in ethanol-fixed populations the GPF fluorescence is destroyed; (D) counterstaining of DNA with propidium iodide (PI) after RNase treatment, revealing the typical cell cycle distribution of growing cells for ethanol-fixed cell samples in contrast to paraformaldehyde-fixed samples.

erties from wild-type GFP. The CX-xGFP used in this study is a red-shifted variant with about 30 times brighter fluorescence than wild-type GFP. This red-shifted spectra of the CX-xGFP variant renders the protein superior to wild-type GFP for fluorescence-activated cell sorting. Maximal emission is achieved using the excitation wavelength of 488 nm, the excitation line of argon lasers, routinely used in many FACS machines.

GFP fluorescence is stable and can be monitored noninvasively in living cells. In paraformaldehyde-fixed cells GFP fluorescence could be maintained, whereas applying fixation protocols using ethanol or methanol,

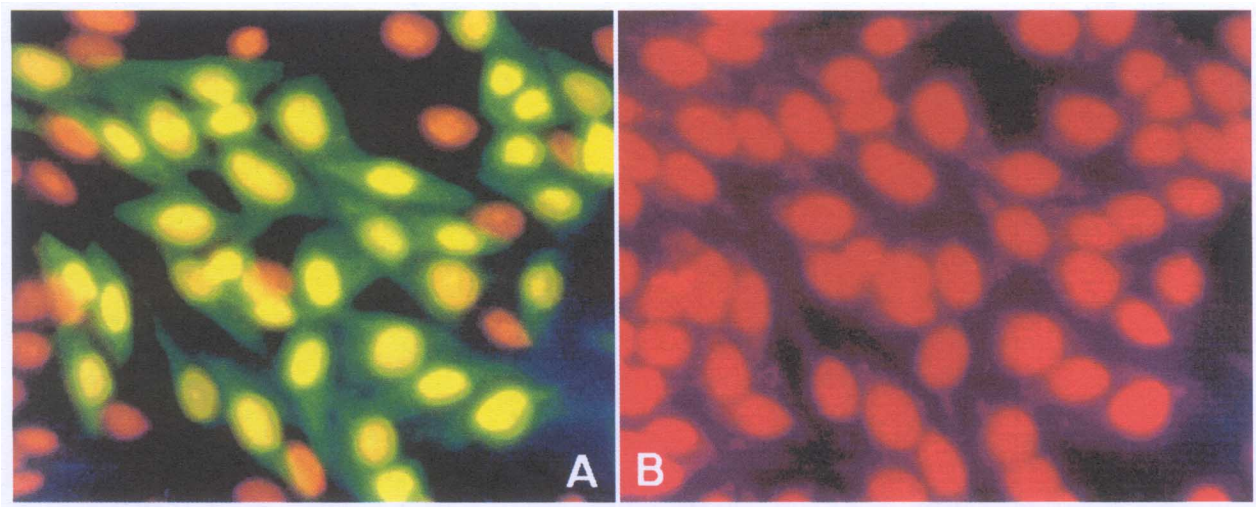

Fig. 4. Monolayer culture of CHO AA8-pGFP clone I. Cells were fixed in 4% paraformaldehyde in PBS for 60 min, washed twice with PBS, digested with RNase for 30 min, and counterstained with propidium iodide (PI). (A) GFP was detected using a filter set suitable for fluorescein detection (excitation, BP 450–490 nm; emission, LP 520 nm). (B) PI-stained DNA was detected using the standard filter combination for this application (excitation, LP 510 nm; emission, LP 590 nm). Due to RNase treatment the amount of GFP-positive cells was reduced to about 40–60% compared to samples where RNA has not been enzymatically digested (see Fig. 2 and 3).

the GPF fluorescence is exhausted, probably due to the fact that the small soluble GFP molecules come out of cells whose plasma membranes have been delipidized. For double-labeling of GFP expressing cells the DNA dye of choice would be either DAPI or Hoechst 33258, both known as DNA-specific probes. The advantage of excitation (355-nm) and emission (455-nm) peaks far apart from the corresponding GFP peaks (488 and 509 nm) is extremely valuable in fluorescence microscopy. For FACS analysis, this advantage turns to a disadvantage, as FACS machines are not routinely equipped with a second laser for excitation. The DNA dye routinely applied for FACS analysis is propidium iodide (PI), which requires complete digestion of RNA for reliable results. RNA digestion and PI fluorescence are usually applied for methanol-fixed or ethanol-fixed preparations; nevertheless, paraformaldehyde fixation followed by RNase digestion and counterstaining with PI gave satisfactory results. Other DNA-specific dyes, like mithramycin and picogreen, cannot be used in combination with GFP due to the fact that all these compounds emit light in the green spectral region.

For using GFP as a marker for cell viability, exponentially growing cells exposed to UVC were harvested after certain postirradiation growth periods, and GFP expression was determined by FACS analysis. From the incubation period of 4 h after irradiation, a loss of GFP was observed in a certain subpopulation with increasing UVC fluences to which the cells were exposed. We suppose that GFP fluorescence in this irradiated subpopulation was not detected due to a leak-out of the soluble GFP molecules, i.e., due to impairment of the cellular

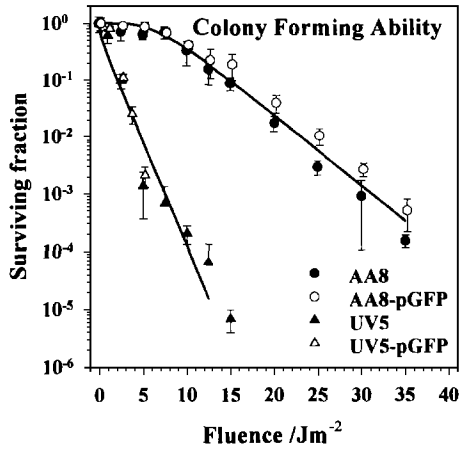

Fig. 5. Survival curves of repair-proficient (AA8) and repair-deficient (UV5) CHO cell strains with and without GFP expression vector pCX-xGFP.

Table I. Radiosensitivity of *in Vitro* Cultivated CHO Cell Clones With and Without GFP Expression Vector pCX-xGFP

Cell line	D_0	n	D_q	r^2
AA8	3.50 ± 0.098	7.32 ± 1.15	6.98 ± 0.72	0.874
AA8-pGFP	3.79 ± 0.086	5.95 ± 0.68	6.77 ± 0.57	0.954
UV5	0.96 ± 0.018	1.10 ± 0.18	0.09 ± 0.16	0.952
UV5-pGFP	0.87 ± 0.020	1.53 ± 0.13	0.34 ± 0.08	0.974

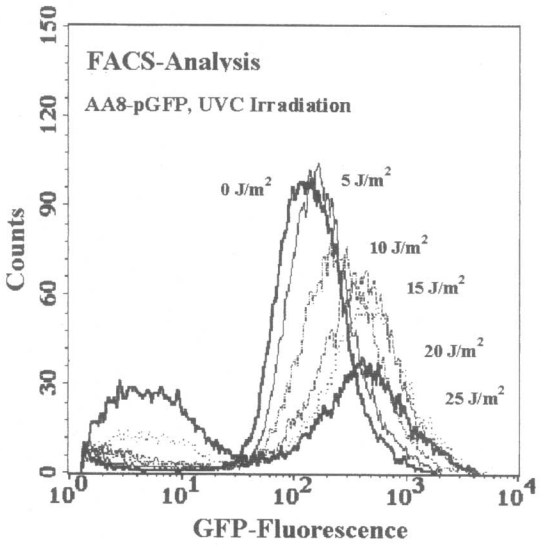

Fig. 6. GFP expression 72 h after UVC (254-nm) irradiation: with increasing dose the fraction of cells not expressing GFP increases slightly (left); in GFP expressing cells the amount of GFP per cell increases (right).

membranes as is the case in apoptotic cells. A second subpopulation was found to enrich GFP in a dose-dependent way, starting from 4 h postirradiation. It is assumed that these cells did not exhibit membrane damage but continued synthesis of proteins (as well as GFP). This effect of irradiation on gene expression driven by constitutive promoters can presumably be explained by progressive protein synthesis of irradiated cells which fail to divide. In experiments inhibiting DNA synthesis by hydroxyurea, an inhibitor of ribonucleotide reductase, a similar effect can be achieved (results not shown).

Experiments describing the effect of ionizing radiation on the expression of the CAT gene under the control of different promoters in transient transfections Cheng and Iliakis [4] demonstrated vectors to be radiation independent or dependent, irrespective of whether the promoters were constitutive or inducible. By use of stable transformats, in which the transfected DNA has been stably integrated into the genome, locus-specific effects might be responsible for complex reactions differing from one cell clone to another. Other authors also found increased gene expression after X-irradiation or UV exposure of stable transfectants [6,9]. For certain viral promoters, the observed up-regulation after irradiation might be a common response with transfected vectors and needs to be considered when radiation-induced responses are studied.

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