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Improving stable transfection efficiency: antioxidants dramatically improve the outgrowth of clones under dominant marker selection

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ABSTRACT

Many cell lines are sensitive to growth at low cell density and undergo apoptosis induced by oxidative stress if the cell density is decreased below a critical threshold. In stable transfection experiments this cell density-dependent growth may be the limiting factor, since during drug selection the cell density falls below the critical threshold, precluding outgrowth of transfected clones. We describe here a simple protocol for the establishment of stably transfected human B cell lines making use of the protective action of antioxidants. The protocol includes: (i) seeding the cells in medium supplemented with sodium pyruvate, α-thioglycerol and bathocuproine disulfonate; (ii) delaying the onset of dominant marker selection to improve recovery of the cells after electroporation. Stably transfected clones have thus been obtained from Burkitt's lymphoma lines, which have been regarded as untransfectable. Using this protocol the stable transfection efficiency with episomal plasmids approaches the transient transfection efficiency, indicating that virtually every transfected cell can be established as a stably transfected clone. This protocol should also prove useful for other cell lines, e.g. neuronal cells, having similar sensitivities to oxidative stress.

The efficiency of establishing stably transfected cell lines is dependent, on the one hand, on the efficiency of gene transfer into a given cell line and, on the other hand, on the survival of successfully transfected cells when a selective pressure is applied. Since the efficiency of gene transfer into a given cell line can be easily determined using reporter plasmids whose gene products can be quantified enzymatically or visualized at the single cell level, most transfection protocols have attempted to improve the efficiency of transfection (e.g. by electroporation; 1–5). Relatively little attention has, however, been devoted to the problem of survival and outgrowth of successfully transfected cell clones under selective conditions, which is the main focus of this work.

Before a cell line can be used for stable transfection and selection with a dominant marker, gene cell density and concentration of the drug have to be titrated against each other to determine the window in which selection is feasible. The lower the cell density, the better the selection at a given drug concentration, and above a critical cell density selection may not work at all. For the Burkitt's lymphoma (BL) line BL2, a cell line particularly critical in this respect, such a standard titration is shown in Figure 1A. It shows that at a cell density of 1280 per well (i.e. 12 800 cells/ml) a hygromycin concentration of at least 200–250 μg/ml and at a cell density of 2560 per well a hygromycin concentration of at least 250–300 μg/ml is required.

For many cell lines cell survival and cell growth are critically dependent on the cell density in culture. In standard tissue culture media, like RPMI 1640 with 10–20% fetal calf serum (FCS), many cell lines do not grow at densities below 100–1000 cells/ml. Some cell lines, like human EBV-negative and EBV-positive (so called group I) BL cell lines are exquisitely sensitive to seeding at low density and undergo apoptosis below a critical cell density of 10 000–50 000 cells/ml (6). For BL2 cells the critical threshold in the experiment shown in Figure 1A is 1280 per well (i.e. 12 800 per ml). Drug selection with a dominant selectable marker kills the non-transfected cells, decreases the cell density below the critical threshold and thus drives the cells into apoptosis irrespective of whether the cells have taken up the dominant selectable marker gene or not. As a consequence, only cell lines that can be easily cloned and resist the selection conditions have been used for stable transfection experiments. In the case of BL cell lines, the feasibility versus non-feasibility of stable transfection experiments has dictated the use of cell lines such as DG75 and BJAB that deviate dramatically in their susceptibility to apoptosis from *bona fide* BL cells (6–8). It has thus been impossible for technical reasons to stably transfect genes involved in the regulation of apoptosis into appropriate target cells more suitable for the study of gene effects.

We have studied the growth properties of BL cell lines in detail and have found that the sensitivity of BL cells to apoptosis is due to their limited ability to take up cystine from the medium (6,8). Since cystine uptake is the rate limiting step for synthesis of glutathione, the cells are unable to cope with increased oxidative

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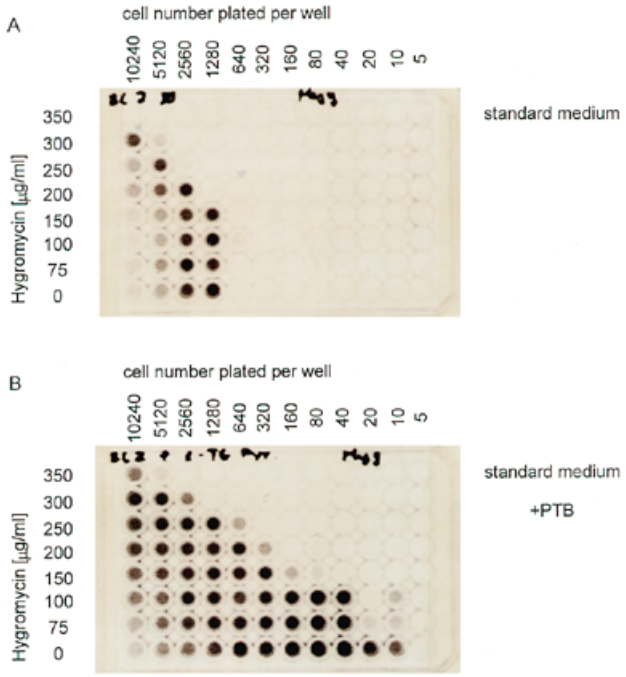

Figure 1. Determining the conditions for drug selection. Untransfected BL2 cells were plated in standard medium (RPMI 1640, 10% FCS, 20 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin) (**A**) or standard medium supplemented with 1 mM sodium pyruvate, 50 µM α-thioglycerol, 20 nM BCS (PTB) (**B**) at the indicated cell densities and hygromycin concentrations. Ten days after seeding, cells were stained with MTT (20) and the plates photographed. Only in wells containing proliferating cells did the yellow color of MTT turn to the blue color of reduced MTT (only the blue color of reduced MTT is visible in the photograph). Drug selection at low cell density is possible only in the presence of PTB.

stress to which they are exposed at low cell density. The cells can, however, grow at low density if a mixture of protective antioxidants is added (8). Taking advantage of this knowledge we reasoned that outgrowth of stably transfected cell clones should be possible if the cells are plated in selective medium under conditions that protect the cells from apoptosis. We used HH514, a subclone of the BL cell line P3HR1, and BL2 as model systems to work out appropriate selection conditions. As shown in Figure 1B, cells can still proliferate if plated at low cell density in the presence of 1 mM sodium pyruvate (9,10), 50 µM α-thioglycerol (α-TG) and 20 nM bathocuproine disulfonate (BCS) (abbreviated to PTB) (8). Figure 1B also shows that lower hygromycin concentrations can be applied if the cells are plated at lower density in the presence of PTB.

HH514 cells have been used successfully in our laboratory in transient transfection assays, but stable transfectants have been difficult to obtain (11,12). Transient transfections using an expression plasmid for green fluorescent protein (GFP) as a reporter gene revealed high transfection efficiencies, exceeding 50% in some experiments. Representative experiments are given in Table 1. The transfection efficiency of HH514 cells is apparently in the same range as that of BJAB and DG75 cells.

As a next step HH514 cells were transfected with an EBV-based episomal vector, BC230A, which was designed for optimized gene expression and expression cloning (13). This vector carries the episomal origin of replication, *oriP*, the *EBNA1* gene required for episomal replication in EBV-negative cells and the regulatory elements of the immunoglobulin κ locus (14) behind an expression cassette (15) which is driven by the human cytomegalovirus immediate early promoter and enhancer. Aliquots of 10^7 cells were electroporated at room temperature with 10 µg BC230A DNA in a total volume of 300 µl RPMI1640 medium without FCS in a 0.4 cm electroporation cuvette applying 260 V and 960 µF. Transfection conditions were chosen to kill 30–50% of the cells. For cells exhibiting low transient transfection efficiencies, voltage, capacity and volume may be modified according to Baum *et al.* (5; Table 1). After electroporation, cells were resuspended in 10 ml RPMI1640 containing 10% FCS and the cells allowed to recover overnight. The following day viable cells were counted and the cells were plated in flat bottom 96-well plates at densities of 100 and 10 cells/well in a total volume of 100 µl RPMI1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin with or without PTB. In the experiment shown in Table 2 hygromycin was added at day 0 or day 6 after plating at a final concentration of 250 µg/ml. Proliferation of cells was monitored microscopically at least twice a week and the experiment finally evaluated after 4–5 weeks. In duplicate plates with 100 cells/well and PTB added proliferation occured in almost all wells and in 26 and 27 wells/plate where 10 cells/well were plated. In contrast, in none of the wells of duplicate plates with 100 and 10 cells/well, were proliferating cells detected in the absence of PTB. Addition of hygromycin on the day of plating reduced the efficiency of clonal outgrowth by a factor of at least 20- to 30-fold (Table 2). The detrimental effect of adding hygromycin imediately after plating could, in part, be compensated for by addition of 5000 untransfected cells to 100 or 10 transfected HH514 cells/well. Addition of untransfected cells, however, could not support survival and proliferation of the transfected cells in the absence of PTB (Table 2).

Table 1. Determining optimal transfection conditions

Cell line	Voltage	Percent survival after electroporation	Percent GFP-positive among surviving cells
DG75	210 V	71	15
	240 V	66	32
	270 V	54	42
	300 V	45	45
HH514	210 V	64	11
	240 V	31	17
	270 V	28	15
	300 V	12	21
BJAB	210 V	55	30
	240 V	31	30
	270 V	25	36
	300 V	11	33

10^7 DG75, HH514 or BJAB cells were electroporated in a 0.4 cm BioRad electroporation cuvette at 960 µF and the voltages indicated with 10 µg GFP expression plasmid in a total volume of 300 µl medium without serum at room temperature. GFP expression and survival were monitored by FACS analysis the day after electroporation. Dead cells were differentiated from living cells by 0.2 µg/ml propidium iodide staining.

Table 2. Antioxidants and delayed onset of drug selection dramatically improve the outgrowth of stably transfected HH514 cells

PTB mix	No. transfected cells plated/well	Addition of untransfected cells	Hygromycin-B added (day)	No. positive wells
+	100	–	6	96/94
–	100	–	6	0/0
+	10	–	6	26/27
–	10	–	6	0/0
+	100	–	0	5/3
–	100	–	0	0/0
+	10	–	0	0/0
–	10	–	0	0/0
+	100	+	0	74/76
–	100	+	0	0/0
+	10	+	0	32/33
–	10	+	0	0/0

HH514 cells were electroporated with plasmid BC230A as described in the text and plated in 96-well plates in the presence or absence of PTB 1 day after electroporation. The number of wells with stably transfected hygromycin-resistant cell clones per 96-well plate is given from two experiments. The presence of the transfected plasmid was verified by Southern blot analysis and all tested clones contained the selectable marker plasmid. Untransfected cells never gave rise to clonal outgrowth during drug selection (data not shown).

Table 3. Antioxidants improve the clonal outgrowth of stably transfected BJAB cells

PTB mix	No. transfected cells plated/well	Addition of untransfected cells	Hygromycin-B added (day)	No. positive wells
+	1000	–	0	96/94
–	1000	–	0	2/0
+	200	–	0	86/89
–	200	–	0	0/0
+	1000	+	0	96/96
–	1000	+	0	61/54
+	200	+	0	96/87
–	200	+	0	27/0

BJAB cells were transfected with plasmid pTG76 and plated in 96-well plates in the presence or absence of PTB 1 day after electroporation. The number of wells with stably transfected hygromycin-resistant cell clones per 96-well plate is given from two experiments. Untransfected cells never gave rise to proliferating cell clones during drug selection (data not shown).

To look for reproducibility of the selection conditions in HH514 cells several independent stable transfection assays were performed with plating of 100, 10 and 1 cell(s)/well. Hygromycin was added between days 4 and 6 after plating, depending on the microscopical evaluation of cell recovery. In 14 independent experiments the efficiency of clonal outgrowth was highly reproducible, varying between 1.7 and 10%. In two experiments the rate of transfection efficiency and clonal outgrowth was exceedingly high (21 and 37%). Extremely high transfection efficiencies have also been observed in some transient transfection experiments.

To compare the efficiency of stable transfection in HH514 cells using episomal versus non-episomal vectors two different episomal (BC230A and BC241A) and a non-episomal vector (pTG76) were included in one experiment. pTG76 is a non-episomal vector conferring hygromycin resistance from an optimized hygromycin expression cassette (16). BC241A is identical to BC230A except that it does not contain the regulatory elements of the Igκ locus. BC241A was only slightly less effective than BC230A (2- to 3-fold), whereas pTG76 was ~500-fold less effective than BC230A.

The EBV-negative BL cell line BL2, which is similarly sensitive to seeding at low density as HH514 cells (Fig. 1A), has also been stably transfected following the same selection protocol using episomal as well as non-episomal vectors.

Compared with HH514 cells it is relatively easy to make stable transfectants from BJAB and DG75 cells. To see whether outgrowth of stably transfected clones of BJAB and DG75 cells was also affected by oxidative stress, stable transfection experiments similar to those described above for HH514 cells were performed with these two cell lines and BC230A and pTG76 as vectors. In contrast to HH514 cells, stable clones were obtained in nearly all experiments, including those in which PTB was omitted. For BJAB cells the efficiency of clonal outgrowth was, however, dramatically increased when PTB was included in the medium. This was particularly apparent when cells were plated at a density of 1000 or less per well in the absence of untransfected cells and hygromycin was added at the time of plating (Table 3). Addition of untransfected cells (Table 3) rescued outgrowth of transfected cells as efficiently as a delay in addition of hygromycin (data not shown), although to a lesser extent than addition of PTB. The overall rate of outgrowth of BC230A-transfected BJAB clones was very high under optimized conditions using PTB (6–14%) and non-episomal vectors were only slightly less efficient (1.5–4%). With DG75 cells transfected clones grew out under all conditions and addition of PTB improved outgrowth only marginally. The rate of clonal outgrowth with episomal vectors varied between 2 and 8% and non-episomal vectors were ~20-fold less efficient (data not shown).

Table 4. Protocol for the generation of stably transfected single cell clones

Day 0
Transfection[a] of 10^7 cells, recovery of the cells overnight in 10 ml standard medium containing PTB[b].
Day 1
Counting of surviving cells and plating at cell numbers of 10 000[c], 1000[c], 100, 10 and 1 cell/well in 96-well plates in 100 µl standard medium containing PTB.
Days 2–4
Microscopical evaluation of cell recovery. The selective drug[d] is added as 10× stock solution in fresh medium when the plated cells have undergone two to three cell divisions.
Days 7–14
Microscopical evaluation of clonal outgrowth. Addition of 50 µl fresh medium containing PTB.
Weeks 3–5
Evaluation of clonal outgrowth. Addition of 50 µl fresh medium containing PTB if medium turns yellow. To obtain single cell clones, cells should be expanded from the plates with the lowest number of clones.

[a]Transfection conditions have to be optimized in transient transfection experiments as shown in Table 1 and described in Baum *et al*. (5).
[b]PTB (final concentrations in medium): 1 mM sodium pyruvate; 50 µM α-thioglycerol (α-TG); 20 nM bathocuproindisulfonic acid disodium salt (BCS). Stock solutions: α-TG, Sigma M-6145, 100% = 11.55 M, stored at room temperature; BCS, FW 564,5 Sigma B-1125, dissolved in water as a 10 mM stock solution, stored at –20°C; sodium pyruvate, Gibco no. 11360-039, 100 mM stock solution in water. Preparation of a ready-to-use 1000× stock solution of α-TG and BCS: add 43.3 µl α-TG (100%) and 20 µl 10 mM BCS stock to 10 ml PBS, filter at 0.2 µm and store in aliquots at –20°C. Sodium pyruvate is added when the medium is prepared. The 1000× stock of α-TG and BCS is added to the medium prior to use.
[c]Only for cells with very low transfection efficiencies.
[d]Drug selection conditions have to be optimized in titration experiments, as shown in Figure 1.

We have shown here that outgrowth of stably tansfected cell clones can be dramatically improved when antioxidants are applied during the selection procedure and when addition of the dominant selectable marker is delayed for a few days until the cells have recovered from the stress of eletroporation. This allows one to obtain transfected cell clones reproducibly and at high frequencies of cell lines that are sensitive to induction of apoptosis at low cell density and that have been virtually resistent to stable transfection up to now. Another advantage of the system is that one can be relatively sure of obtaining single cell clones and not mixtures of transfectants, provided the number of positive wells per plate is low. Using episomal vectors almost every successfully transfected cell which survives can be established as a transfected clone. Table 4 summarizes our protocol optimized for BL cells, which are exquisitely sensitive to induction of apoptosis by low cell density. Since cell density-dependent growth and sensitivity to oxidative stress are features not only of human B cells, it is likely that this protocol will turn out to be generally useful for gene transfer into many types of cells, in particular other cells of hematopoetic origin and neuronal cells (17–19).

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