Research Report

Robotic High-Throughput Assay for Isolating Apoptosis-Inducing Genes

BioTechniques 32:670-677 (March 2002)

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ABSTRACT

The need to assign functions to genes will increase in upcoming years because of the accumulation of sequence information from various organisms. Here we present a genetic screen for the systematic functional analysis of genes in mammalian cells. The screen is based on high-throughput, transient transfections of individual cDNAs by a robotic system. This construction uses a moveable pipetting head with a novel 96-channel parallel tube-pump array and an integrated de-lidding device. Because of the robot design, the processing speed can reach 2000 transfections/h. We demonstrate the suitability of the screen to isolate apoptosis-inducing genes as a first read-out. This is achieved in combination with co-transfected reporter plasmids to produce a novel and convenient assay for apoptosis induction.

INTRODUCTION

As progress continues in the sequencing and annotation of the human genome, studies to link sequence information to functions will become of prime importance. However, the functional investigations of genes are likely to take much longer than the mere determination of the sequence. Consequently, systematic high-throughput assays for the determination of gene functions are required. Here we describe an assay that allows the investigation of the activity of individual cDNAs of an expression library for a given cellular function. This screen is straightforward, is performed in intact cells, and is easily adaptable to automation.

We have chosen apoptosis as a first read-out. Apoptosis is the genetically determined cell death. It constitutes a cellular response with wide-ranging consequences, both pathological and physiological, as it plays a role in many different cellular processes (13). In our assay, we use two novel read-outs for apoptosis induction that are based on co-transfected reporter plasmids.

MATERIALS AND METHODS

Cell Transfections

Baby hamster kidney (BHK) cells in 24-well microplates were transfected with 2 μ g plasmid DNA by the calcium phosphate co-precipitation method de-

scribed previously (11). To this end, 25 μ L DNA solution were mixed with 25 μ L 2 $\times$ HBS (pH 6.9; 4°C) and 71 mM M CaCl₂ solution (4°C). The mixture (38 μ L) was distributed onto cells after incubation for 25 min at room temperature. 293T cells were transfected by the addition of 100 μ L 2 $\times$ HBS to a 100- μ L DNA solution (2 μ g apoptosis inducer or empty plasmid and 400 ng reporter plasmid), after which 119 mM CaCl₂ were added. This solution (50 μ L) was added to the 293T cells in the 24-well plates. For the transfections in 293T cells, chloroquine (40 μ M) was given to the medium that was changed 5 h later. We co-transfected the constitutive active secreted alkaline phosphatase (SEAP) plasmid Aptag-1 (3). SEAP activity was measured as described (2).

Determination of β -Galactosidase Activity

We used a β -galactosidase construct under the control of the constitutively active long terminal repeat of the Moloney murine leukemia virus (10). After the transfection, the medium was changed to DMEM without phenol red (Sigma, Deisenhofen, Germany). The enzymatic activity was measured by adding 1/5 volume of a solution of 2.86 mM chlorophenolred- β -D galacto-pyranoside (CPRG) (Roche Diagnostics, Penzberg, Germany), 1 mM MgCl₂, 42.6 mM mercaptoethanol in 0.1 M sodium phosphate to the cells for a final concentration of 0.57 mM CPRG.

Robotic Transfection Protocol

The pipetting head of the robot is mounted on a gantry x-y-z motion system (ERO Führungen, Löffingen, Germany) and controlled by a Microsoft® Windows® NT-based personal computer (e.g., Figure 2).

The transfections were performed in a microplate using the calcium phosphate co-precipitation method and were subsequently added to the cells in the 96-well plates. The process starts with the transfer of the DNA (1 µg plasmid DNA in 20 µL water) into the mixing plates. The solutions L1 (77 mM CaCl₂) and L2 (25 µL 2× HBS) were successively added. After a 20-min incubation, 375 mM chloroquine solution were added and incubated for 3 min, after which the solution was mixed by pipetting 35 µL up and down twice. Aliquots of 12 µL were then slowly added to the microplates with the cells. If BHK cells

were transfected, then the addition of chloroquine was omitted. The transfection of 192 DNA samples into 192 cell cultures were handled in rows.

RESULTS

β-Galactosidase as a Reporter Enzyme to Detect Proapoptotic Genes

Because caspases and proapoptotic cysteine proteases are involved in many but not all kinds of apoptosis induction (4,8), we wanted to use markers of cell death other than the activation of these proteases. Thus, we explored the use of established reporter enzymes that have a proven record of robustness and the advantage that there are many different substrates described. The first apoptosis reporter assay is based on the obser-

vation that, while the overall integrity of the plasma membrane is not altered in apoptosis, the membrane becomes permeable for low molecular weight substances (12). Therefore, we reasoned that substrates for enzymes such as β-galactosidase could enter apoptotic cells, be metabolized, and be detected by an optical read-out. To test this, we have transfected 293T cells with a constitutively active expression plasmid for β-galactosidase together with the apoptosis inducer ANT-1 (1) or an empty control vector. As Figure 1A shows, on the addition of the substrate CPRG to the medium, the wells with apoptotic cells displayed a greater than 5-fold increase in the turnover of the substrate in comparison with the control-transfected cells. A similar effect was seen when this experiment was performed in MCF7 cells (data not shown). To verify our hypothesis that the CPRG substrate that entered the dy-

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ing cells rather than the enzyme was released from these cells, we saved the supernatant. Upon its incubation, we did not observe any further turnover of the substrate (data not shown). To test whether the cells in the CPRG-negative wells efficiently took up the reporter DNA, the transfection efficiency was controlled by a subsequent Triton® X-100 lysis. This released the enzyme from the cells and led to a metabolism of the substrate even in samples without apoptosis induction (Figure 1A). This resulted in an equal increase in the A_{590} between ANT-1-transfected cells ($\Delta 0.36$) and the control-transfected cells ($\Delta 0.41$) in the same time period.

Secreted Alkaline Phosphatase Activity for Detecting Apoptosis-Inducing Genes

One of the hallmarks of apoptosis is the dilation of the endoplasmic reticulum and the eventual dissolution of cytoplasmic organelles (14) that impede the secretion of proteins from the cell. This led us to speculate that the reduced secretion of the well-known reporter enzyme SEAP, under control of a constitutive promoter, would be a suitable indicator of apoptosis induction. Because the plasma membrane is kept intact for high molecular weight substances (6) during apoptosis, proteins would not leak out of apoptotic cells and SEAP would not appear in the supernatant of transfected cells. Furthermore, we employed the fact that the action of pro-apoptotic genes requires the activation of downstream signals and therefore sets in with a temporal delay compared with the secretion of the co-transfected SEAP. SEAP allows one to measure the enzymatic activity at successive time points, since the secreting cells are not fixed or otherwise destroyed. Consequently, we started to quantify the SEAP activity after 16 h after transfection. This initial measurement was used to normalize the succeeding activities and was set to zero to account for the different transfection efficiencies. A time course experiment showed that the difference between samples with and without apoptosis induction reached a plateau after 36 h. Therefore, a second SEAP measurement was performed after 36 h, when

apoptosis induction had resulted in a block in the secretion of proteins and a reduced SEAP activity. Figure 1B shows a 10-fold reduction in SEAP activity in comparison with control-transfected cells when the apoptosis-inducing gene ANT-1 (1) was co-transfected. We have also observed the reduction in SEAP activity on apoptosis induction in HeLa cells. From our experience with this screen, we have found that a 50% reduction in SEAP activity is already a useful indication for apoptosis induction.

Transfection Robot

Since individual cDNAs are investigated in this screen, one has to perform as many transfections as there are independent clones in a library. Because even a normalized cDNA library comprises several hundred thousand clones, the screen requires the use of automatic processing. Commercially available standard robots are unable to achieve the targeted 20 000 transfections/day. Dedicated high-throughput robots offered from specialized manufacturers

Figure 1. Use of co-transfected reporter plasmids to screen for proapoptotic genes. (A) Detection of apoptosis induction by β -galactosidase. 293T cells were transfected in 24-well plates with 0.1 μ g β -galactosidase plasmid and 0.5 μ g ANT-1 or the vector control pHA. After 24 h, the substrate CPRG was added and incubated for 2.5 h. After the A_{590} was determined, the cells were lysed by the addition of 0.2% Triton X-100 for 30 min. The means and standard deviations of three independent experiments are shown for each condition. (B) Measuring SEAP activities to detect apoptosis-inducing genes. BHK cells were transfected with a SEAP expression vector and a control vector or the pro-apoptotic gene ANT-1 (0.5 μ g each). SEAP activity was determined 16 and 36 h after transfection. The SEAP levels 16 h after transfection were used to normalize the activity after 36 h. The means and standard deviations are shown from three independent experiments.

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are extremely expensive and exceed by far the budget of a scientific group.

To establish a fast and less costly alternative to existing pipetting heads, we developed a 96-fold head that is based on the linear tube-pump principle. The basic function of the head is shown in Figure 2A. A set of parallel silicone tubes is mounted on a linear tube holder. A roller is connected to an up-and-down moveable slide and a pneumatic element that can press the roller onto the tubes until they are closed. Fluid is taken up by moving the pressed roller

upward, which allows the tubes to work like syringes with pistons. The upper ends of the tubes are connected to a pressure/suction vessel. The pressure in this vessel is controlled by four valves (Figure 2, V1–V4). For “tube-pump pipetting”, the vessel is connected to 0.02 bar pressure. By releasing the roller from the tubes, this pressure blows out the content of the pipetting tips into the wells without forming droplets at the tips. Alternatively, in the “suction-pressure” mode, the uptake and release of fluid is controlled by the

pressure in the vessel and the releasing time of the roller, which works as a multi-valve. This facilitates the uptake of large volumes up to the milliliter range or the distribution of small or large volumes from the fluid-filled vessel. This supports the use of the head in various protocols (e.g., when the cell culture medium must be changed).

The 96-tube pipetting head (Figure 2B) was built with four tube-holder plates of 24 tubes in a parallel array together with four simultaneously operating rollers and was mounted on the z-slide of the gantry robot system. The bottom ends of the tubes are connected to commercial pipet tips that are arranged in the format of a 96-well microplate. This pipetting head is fully movable over the complete range of the area that is covered by the microplates. The pipetting volume range in the linear tube-pump mode is 5–100 $\mu\text{L}/\text{channel}$. The head works above 10 μL with an accuracy of less than 5% standard deviation, as tested in several pipetting trials with distilled water (Figure 2C). A very similar result was obtained when cell culture medium with 10% FCS was used (data not shown).

The gantry robot system has a platform size of 140 $\times$ 90 cm that provides space for 60 96-well microplates. The plates are arranged in a row and column structure that makes the identification of the plates easy and allows one to work without bar codes. The plates are deposited into subplatforms. The first subplatform (Figure 2B, left-hand side) is cooled to 4°C and takes up the 20 DNA-containing plates. The second subplatform comprises 20 microplates for preparing, mixing, and incubating the DNA before transfection. The third platform (Figure 2B, right-hand side) can be heated to 37°C and holds the microplates with the cell cultures. The vessels that contain the transfection fluids and the automatic tip wash station are placed between the first and the second subplatform. The washer consists of 96 vertically mounted plastic tubes arranged in the format of a 96-well microplate. The pipetting tips are immersed into the tubes to clean them inside and outside.

The cell culture plates on the platform are covered by protection lids that have to be removed for transfection.

Figure 2. The robotic system for high-throughput transfection of genes. (A) Schematic drawing of the pipetting principle using a linear tube-pump combined with a pressure/suction vessel. (B) The robotic platform and the 96-fold pipetting head. See text for detailed descriptions. (C) Accuracy of the pipetting head. A 96-fold parallel pipetting test is shown with distilled water. For the uptake of the fluid, the pressed roller was moved 25.94 mm up, and the water was blown out by 0.02 bar into 96 cups (Eppendorf Scientific, Westbury, NY, USA) whose contents were then gravimetrically determined. The mean over the 96 pipetted volumina is 25.2 μL , SD = 1.95%. A typical result is shown.

tion. Consequently, a de-lidding device with a vacuum cup was integrated into the pipetting head. The lids are moved with the head and repositioned on their microplates immediately after the transfection.

Commercial robot systems that take

and replace microplates from and into an incubator lose a considerable amount of time transporting the plates. In contrast, the platform of our robot system provides enough space for 60 laid-out 96-well microplates. The movable pipetting head has direct access to all

these plates at any time. Thus, by optimally interlacing the pipetting and incubation processes of the protocol, the system is nearly in permanent full-speed operation. A platform of 1920 cDNAs can be processed in 45 min (i.e., a mean transfection throughput of about 2000 samples/h or 40 000–50 000 samples/24 h can be reached). We will now try to design a DNA-isolation robot that approaches this number of DNA preparations in the same time period.

Test of the Reporter Plasmids with the Robotic System

We co-transfected the reporter plasmids together with a control vector or the apoptosis inducing-gene ANT-1 using the transfection robot. Figure 3A demonstrates that the β -galactosidase activity assay allowed the detection of four wells in a 96-well plate in which the proapoptotic ANT-1 plasmid was co-transfected (Figure 3, indicated by an asterisk). After the addition of CPRG, the A_{590} of between 0.81 and 0.98 were clearly distinguishable from the wells without an apoptosis inducer, which had a mean A_{590} of 0.32 [z-value 0.49 (18)]. Triton X-100 lysis revealed that the cells in the CPRG-negative wells had nevertheless been efficiently transfected with the reporter plasmid (Figure 3B). Figure 3C demonstrates that we were also able to detect the four samples that contained the apoptosis inducer using the secreted alkaline phosphatase reporter plasmid. Its activity was repressed to an A_{405} of between 0.11 and 0.05 in those wells in which the ANT-1 plasmid was co-transfected, whereas the average A_{405} was 0.46 in the rest of the samples (z-value 0.34).

DISCUSSION

In a previous work, we have described the isolation of plasmid DNAs for multiple transfections (9). The present study details their use in a genetic screen for the functional isolation of genes. To this end, individual plasmid DNAs from a cDNA library are transfected into mammalian cells by a 96-well robotic head. The cellular function of each of these genes is then tested

Figure 3. High-throughput analysis of apoptosis-inducing genes using the transfection robot and reporter plasmids. (A and B) Determination of apoptosis-inducing genes with a β -galactosidase reporter plasmid in a 96-well format. The robotic system was used to transfect 96 samples into 293T cells. The four wells to which the plasmid for ANT-1 (150 ng) was added are marked with asterisks; the rest of the wells received 150 ng of an empty control vector. After the transfection (20 h), the β -galactosidase substrate CPRG was added, and the A_{590} was determined (Figure 3A). Triton X-100 lysis indicates equal transfection efficiencies (Figure 3B). The cells in each well were solubilized by the addition of Triton X-100 (0.2%), and the activities of the β -galactosidase enzyme were measured by the A_{590} in an ELISA reader. (C) Use of secreted alkaline phosphatase as a reporter assay to detect proapoptotic genes in a 96-well format. The SEAP plasmid (72 ng) and 180 ng of an expression plasmid for ANT-1 (*) or an empty vector were transfected into BHK cells by the transfection robot. The supernatant (18 and 30 h later) was tested for the secreted alkaline phosphatase activity at an A_{405} in an ELISA reader.

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with an appropriate assay based on co-transfected reporter plasmids.

The newly constructed robot is tailored to the task of transfecting cells at high throughput. For this purpose, the robot design has a linear arrangement of the microplates that makes the plates easily accessible for the pipetting head and speeds up the parallel transfections. Furthermore, the robot displays a novel 96-fold pipetting head to accommodate different transfection protocols.

The screen achieves a superior sensitivity in comparison with other expression cloning assays because the cellular effect can be measured in a population of cells, each transfected with the same gene. This facilitates the detection of gene functions that were inaccessible with conventional approaches, such as the degenerative process of apoptosis induction that was used as a first read-out. Because only a subpopulation of the cells in each well are transfected,

the assay for a cellular function requires the establishment of suitable reporter plasmids. The development of reporter constructs for apoptosis is especially demanding, as it is a degenerative cellular process. Various reporter constructs that indicate the activation of the proapoptotic enzyme family of the caspases using the fluorescence resonance energy transfer (FRET) effect have been described (7,15). However, this assay requires a sensitive fluorescence reader and has not yet been successfully demonstrated in a high-throughput format. In addition, we speculated that some genes induce apoptosis so rapidly that not enough GFP protein is synthesized for its detection. Consequently, we restored the enzymes as sensitive reporters. Both reporter plasmids, SEAP and β -galactosidase, are used here to measure cellular alterations that are the general consequences of apoptosis induction. The β -galactosidase read-out

employs the increased permeability of the membrane of apoptotic cells for low molecular weight substances. SEAP, on the other hand, uses the reduced secretion of proteins and the impermeability of the membrane of apoptotic cells for proteins.

Both reporters require two measurements of the enzymatic activity. With SEAP, the first measurement indicates the transfection efficiency; with β -galactosidase, the second measurement detects the uptake of the reporter DNA. While the β -galactosidase read-out only requires the addition of the CPRG substrate, the handling of the SEAP assay is more elaborate because the supernatant has to be isolated and further processed. However, the SEAP read-out has the advantage that it allows additional measurements at later time points to detect slow apoptosis inducers. On the other hand, a reduced secretion of alkaline phosphatase could also

possibly be achieved by genes that cut off the secretory pathway. β -galactosidase might be a more specific read-out because only few genes are able to change the permeability of the plasma membrane. Therefore, the two read-outs complement each other as they measure different parameters of apoptosis and might therefore be suitable to be used in combination to verify the apoptosis-inducing genes.

Genes isolated with this screen define processes in the cell whose disturbance leads to apoptosis. Because apoptosis induction plays a role in many different processes, these activities might be involved in physiological or pathological cell death induction. Under which circumstances and how the isolated genes induce the process of apoptosis have to be determined in every case. Strikingly, most of the genes that mediate the signal for apoptosis induction also have the activity to induce apoptosis on overexpression. This might be explained by the observation that protein-protein interactions are largely responsible for the induction of apoptosis (16,17). On overexpression, these interactions are generated and apoptosis is induced. Therefore, the screen for pro-apoptotic genes has to be regarded as a means to isolate genes that have to be further investigated to integrate them into pro-apoptotic signalling pathways, as we have shown previously (5).

Probably many if not most gene activities cannot be used for selection because they can only be activated transiently. Otherwise, they would impede the growth of the cell. Consequently, various versions of this screen for other dominant gene activities are conceivable, such as for the inhibition of the cell cycle, antisense, RNA interference effects, the induction of differentiation, or protein translocation. Many other methods, some of them more expensive than the ones described here, can also be integrated into the screen, such as the use of lipofection for the introduction of DNA, fluorescence, and flow cytometry in the read-out. Therefore, we think that systematically investigating the effects of single cDNAs in a transient transfection screen, as described in this study, holds a certain potential for the functional investigation of gene sequences.

ACKNOWLEDGMENTS

This work was supported by the Bavarian Government, Xantos Biomedicine AG, and Roche Diagnostics.

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Received 6 December 2001; accepted 22 January 2002.

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