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Received 22 August 2003; accepted 19 November 2003.

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High cell density induces expression from the carbonic anhydrase 9 promoter

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BioTechniques 36:228-234 (February 2004)

Efficient ways to control the level and timing of the expression of specific genes in cultured cells and tissues, including tumors, have received considerable attention (1,2). Inducible expression systems should have a minimal activity in the basal state but should allow rapid accumulation of the heterologous protein upon stimulation. The most advanced inducible systems employ combinations of functional domains from prokaryotic, eukaryotic, or viral proteins to create chimeric transactivators capable of modulating gene expression in a drug-dependent manner (1). The second component in these systems is an inducible promoter, which consists of a multimerized transactivator binding sequence linked upstream of a minimal promoter. In the presence of inducer, the chimeric activator binds specifically to its DNA recognition sequence and activates the transcription of the target gene (1). Although very specific and effective, these chimeric systems usually require specialized cell lines or have to be prepared in several relatively time-consuming steps. Therefore, it may be advantageous to use systems that are easier to generate and yet retain significant inducibility. Here we describe the cell density-dependent activity of the carbonic anhydrase 9 (CA9) promoter and propose its utility as an inducible expression system.

The expression of carbonic anhydrase IX (CAIX, previously known as MN) has been detected in a large number of carcinomas and carcinoma-derived cell lines but not in the corresponding normal tissues (References 3, 4, and references therein). The mechanism of CAIX induction in dense cultures was the subject of our previous study (5). Earlier, oxygen levels in sparse (10⁶ cells in 100-mm plates)

and dense (10⁶ cells in 34.8-mm plates) LNCaP human prostate cells were established as 13% (96 mmHg) and 9% (70 mmHg), respectively (6). Reoxygenation by stirring abrogated CAIX expression, suggesting that CAIX expression in cultured cells is indeed triggered by an intermediate decrease of O₂ tension due to increased O₂ consumption and not by cell contacts per se. This decreased O₂ tension, also termed pericellular hypoxia (6), is too high for an appreciable stabilization of hypoxia-inducible factor 1 α (HIF-1 α), but it is sufficient for the activation of a phosphatidylinositol 3'-kinase (PI3-K)-dependent pathway (5). Earlier studies defined the CA9 promoter in the (-173; +31) region (the numbers in parentheses indicate each position relative to the transcription start), which appears to contain the critical regulatory elements for CA9 transcriptional activation (7). Among these, the hypoxia-response element (HRE) (8) and SP1/SP3 binding protected region 1 (PR1) (9) are crucial for CA9 transcriptional activity.

The striking effect of cell density on CAIX expression prompted us to investigate the utility of the CA9 promoter as a cell density-inducible expression system. The (-173; +31) CA9 promoter fragment was cloned in the pGL2-Basic (Promega, Madison, WI, USA) and pEGFP-1 (BD Biosciences Clontech, Palo Alto, CA, USA) vectors. The (-2361; +298) vascular endothelial growth factor (VEGF) gene fragment was also cloned in the pGL2-Basic vector. The simian virus 40 (SV40) early promoter-driven pGl2 control vector was obtained from Promega. To prevent the possible modulation of CA9 promoter activity by the SV40 promoter/enhancer sequence present in pEGFP-1 (GenBank[®] accession no. U55761; positions 1694–1925),

this sequence was deleted. The human cervical carcinoma HeLa and osteosarcoma Saos-2 cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (Cambrex, Baltimore, MD, USA), supplemented with 10% fetal calf serum (FCS) (Invitrogen, Carlsbad, CA, USA), 1.10^2 U/mL penicillin (Sigma, St. Louis, MO, USA), 1.10^2 μ g/mL streptomycin (ICN Biomedicals, Costa Mesa, CA, USA), and 125 ng/mL amphotericin B (Sigma). The cells were transiently transfected with a pGL2 construct (expressing firefly luciferase) and pRL-CMV (expressing *Renilla* luciferase) (Promega) using the Effectene[®] kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Following an 8-h exposure to the transfection mixture, the cells were rinsed with phosphate-buffered saline (PBS), trypsinized, replated at varying densities, and harvested at the indicated times. The firefly and *Renilla* luciferase activities were assayed with the Dual-Luciferase[®] Reporter Assay System (Promega) in the Monolight[™] 2010 luminometer (BD Biosciences, San Jose, CA, USA). Promoter activity was expressed as the average of ratios of firefly to *Renilla* luciferase activities from three independent experiments. For the green fluorescent protein (GFP) experiments, HeLa cells were cotransfected with the pEGFP-1 construct containing the CA9 fragment and pCEP4 (conferring resistance to hygromycin) (Invitrogen) at a 10:1 ratio. The stably transfected cells were selected in the presence of 400 U hygromycin/mL media for 3 weeks. The mass population of transfected cells was plated at 20,000 and 160,000/cm² and tested for fluorescence 24 h later.

Initially, we investigated the time course of the cell density-dependent induction of the CA9 promoter activity in transiently transfected HeLa cells. The transfected cells were replated at varying densities and harvested after 24, 48, and 72 h incubation. After 24 h, a basal activity of the CA9 promoter was observed in all tested densities, except 160,000/cm², where a 3-fold induction was observed (Figure 1A). Forty-eight hours after replating, a moderate induction was observed in cells plated at 40,000 and 80,000/cm² (2.5- and 3.3-fold, respectively), whereas a 9-fold in-

duction was observed in cells plated at 160,000/cm² (Figure 1A). The most efficient induction was observed after 72 h. The activity of the CA9 promoter in cells plated at 40,000 and 80,000/cm² increased 4- and 6-fold, respectively,

and a more than 15-fold induction was observed in the cells plated at 160,000/cm² (Figure 1A). These observations confirm the strong dependence of CA9 promoter activity on the density of replated cells (transfection efficiency

Figure 1. Cell density-dependent activity of the carbonic anhydrase 9 (CA9) promoter. (A) Luciferase activity in transiently transfected HeLa cells. HeLa cells were cotransfected with the (-173; +31) CA9 promoter fragment in pGL2-Basic and pRL-CMV. Following an 8-h exposure to the transfection mixture, the cells were trypsinized and plated. The cells were then harvested, and the reporter assay was performed. The activity of the CA9 promoter is expressed as the ratio of firefly to *Renilla* activity for each density and time point. Each of the points represents the mean value ($\bar{x} \pm$ SD) from at least three individual experiments. (B) Luciferase activity generated from the CA9, vascular endothelial growth factor (VEGF), and simian virus 40 (SV40) promoters in transiently transfected Saos-2 cells. Saos-2 cells were transfected as in panel A. The cells were harvested 24 h after replating at 20,000 and 160,000/cm². The activity of each promoter is expressed as the fold increase of the ratio of firefly to *Renilla* activity in dense (160,000/cm²) over sparse (20,000/cm²) cultures. (C) Green fluorescent protein (GFP) activity in stably transfected HeLa cells. HeLa cells were cotransfected with the (-173; +31) CA9 promoter fragment in pEGFP-1 and pCEP4 at a 10:1 ratio. The mass population of the stably transfected cells was plated at 20,000 and 160,000/cm² and tested for fluorescence 24 h later. A representative photograph is shown for each cell density (magnification: 100 $\times$). AU, arbitrary unit.

was the same for all cells because the same transfection mixture was used). Basal activity (slightly above the background) in sparsely plated cells (20,000/cm²) indicates the tight control of the *CA9* promoter activity. Similar results were obtained in a number of other cell lines, such as Saos-2, HT 1080 (fibrosarcoma), MCF-7 (breast carcinoma), M006 (glioblastoma), and SiHa and CaSki (cervical carcinoma) (data not shown).

VEGF expression is also regulated by cell density (10,11), although the regulatory elements involved have not been identified. Therefore, we wanted to relate the cell density-dependent induction of the *CA9* and VEGF promoters. Transiently transfected Saos-2 cells were harvested 24 h after replating at 20,000 and 160,000/cm². Reporter activities indicated about a 12-fold increase of the *CA9* promoter activity in dense cultures, compared to about a 3-fold increase of VEGF promoter activ-

ity (Figure 1B). Increasing cell density had no effect on the SV40 promoter activity (Figure 1B). Thus, a direct comparison of the *CA9* and VEGF promoters revealed a substantially higher induction of the *CA9* promoter under conditions of increasing cell density.

To further probe the efficiency of the *CA9* promoter in controlling cell density-dependent expression, HeLa cells were stably transfected with the GFP vector. In agreement with the results of transient transfections, the activity of the GFP was also dependent on the initial density of the plated cells. No fluorescence was observed in cells plated at 20,000/cm², whereas significant fluorescence was observed in cells plated at 160,000/cm² (Figure 1C). The fluorescence observed was extensive, but focal areas of low expression were noticed. This heterogeneous fluorescent pattern is not the consequence of using a mass population of stably transfected cells because similar results were ob-

served with several individual clones (data not shown). In our opinion, this irregular expression pattern reflects the heterogeneity of conditions (with respect to decreased O₂ tension) prevailing in dense cultures. Although it would seem that the cells in culture should be exposed to the same conditions, in fact, the cells throughout the high cell density culture experience varying microenvironmental conditions, and we believe that the *CA9* promoter functions as a sensor of the heterogeneity of O₂ levels in this environment.

Transformed cells, unlike their normal counterparts, do not display density-dependent inhibition of cell division. As a result, they do not stop dividing after a confluent monolayer is formed and instead produce increasingly denser cultures by multilayering. This increasing density is associated with alterations in the expression patterns of multiple genes. Although several mechanisms responsible for the

cell density-dependent modulation of gene expression have been proposed [e.g., involvement of soluble factor(s), changes in cell shape, and direct cell-cell contacts or their combination] (10), in the case of the *CA9* promoter, the primary cause is the decreased O_2 tension in dense cultures due to increased O_2 consumption (5).

There has recently been considerable progress in dissecting the *CA9* promoter function (5,7–9). The cooperation between transcription factors binding HRE and PR1 is critical for the inducibility of the *CA9* promoter by cell density (5), while the remaining positive regulatory elements amplify the signal. In the absence of an inducing signal, the activity of the *CA9* promoter is repressed by the silencer element PR4, which leads to the tight control of basal expression (7).

Using two different reporters, we were able to demonstrate the tight regulation of the *CA9* promoter by cell density and associated lower O_2 tension in several cell lines. Apparently, the regulation of the *CA9* promoter by cell density in transformed cell lines is a general phenomenon, and we therefore hypothesize that the *CA9* promoter will display cell density-dependent activity in most cells that are capable of reaching a certain density that will generate mild hypoxia. The only requirement is that the cells must be HIF-1 α , SP1/SP3, and PI3-K competent (5). Indeed, we have shown that many different types of malignant cells behave in this fashion.

Induction by cell density does not involve treatment with an exogenous agent and no special conditions are required, except for replating cells transfected with the expression construct under the control of the *CA9* promoter at high density. The proposed cell density-inducible system utilizing the *CA9* promoter thus represents an easy and readily applicable alternative to other inducible expression systems.

The unique organization of the *CA9* promoter (HRE immediately upstream of transcription start) results in tight control under normoxic conditions (21% O_2) and efficient induction under conditions of mild to strong hypoxia (5%–0.5% O_2). The *CA9* promoter is therefore an excellent candidate for var-

ious O_2 -sensing applications, including the monitoring of hypoxia in vivo or in vitro or the expression of suicide genes in hypoxic tumors. It is possible that combinations of multiple HRE and PR1 regulatory elements in front of the *CA9* or a heterologous promoter will be more efficient for directing cell density-dependent expression and will yield more homogenous expression than the wild-type *CA9* promoter.

ACKNOWLEDGMENTS

This study was supported by a grant from the California Cancer Research Program (00-00789V-20240) and The Avon Foundation. The (-2361; +298) VEGF gene fragment was kindly provided by Dr. Chris Hughes (Department of Biochemistry and Molecular Biology, University of California, Irvine, CA).

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Received 28 October 2003; accepted 18 November 2003.

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