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Regulation of cell specific expression of calcyclin (S100A6) in nerve cells and other tissues

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Abstract. Many of the small, acidic, calcium binding S100 proteins present in the brain specifically map different anatomical regions and cell types and their overexpression is implicated in pathological changes. Similarly to other members of the S100 protein family, calcyclin (S100A6) is expressed in a cell specific manner and is found in subpopulations of neurons and astrocytes in the brain and in epithelial cells and fibroblasts. In this article we review data concerning the cell specific expression of S100 protein genes and present experimental results on the regulation of the calcyclin gene. We have performed promoter deletion studies to locate regions within the calcyclin gene promoter responsible for transcriptional regulation. The results demonstrate that the 3 kb long calcyclin gene promoter lacks a cell specific cis-acting element and drives the expression of the reporter gene also in cells that do not express endogenous calcyclin. The expression is modulated by positive and negative elements acting uniformly in the four different cell lines studied. The first intron of the calcyclin gene was found to have an inhibitory influence on expression regardless of cell type. It was also shown that calcyclin expression can be induced in calcyclin-negative cells by treatment with 5-azacytidine suggesting the involvement of gene methylation in its cell specific expression. The results are discussed in light of the data available on the regulation of other S100 genes.

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GENERAL CHARACTERISTICS OF S100 PROTEINS

The S-100 family of proteins groups small (10 kDa), highly homologous, acidic calcium binding proteins containing two EF hands motifs (Zimmer et al. 1995). Calcium binding is a structural and functional feature defining the biological role of these proteins as molecules capable of switching the change in cellular calcium concentration into modulation of cellular processes. In the case of some S100 proteins the molecular interactions in which they are involved have been described while for others, less characterized, the biological role can only be speculated on the basis of the effects of their presence or overexpression in a cell (reviewed in: Zimmer et al. 1995, Schafer and Heizmann 1996).

It was shown that in man most of the genes coding for the S100 proteins are localized in a sequence on the first chromosome (Schafer et al. 1995). Three of these genes have also been found to form a cluster in mice chromosome 3 (Dorin et al. 1990). The location of the S100 genes on the human chromosome 1 served to systemize the proteins by numbering them according to the position of their genes: S100A1, S100A2 etc. while proteins whose genes are located elsewhere preserved their original or customary names, for example S100B. Most of the S100 genes consist of three exons, the first of which is not translated (Engelkamp et al. 1993). The coding sequences are highly homologous but the sequence of introns and promoters does not show much resemblance. There is only one 12-nucleotide long sequence in the proximal promoter that is shared by most of the S100 genes (Allore et al. 1990) but its functional significance, if any, has not been described.

An interesting feature of the S100 proteins is that they are expressed in a cell specific way. For example the expression of S100A8 and S100A9 proteins is limited to granulocytes and monocytes (Roth et al. 1994), S100A6 (calcyclin) is characteristic for epithelial cells and fibroblasts (Kuźnicki et al. 1992), while S100B is typical for glial cells. In brain S100A1 predominantly maps the neurons, S100B is abundant in glial cells (Song and Zimmer 1995) while S100A6 is found in subpopulations of neurons and astrocytes (Yamashita et al. 1999). Overexpression of some S100 proteins often correlates with pathological changes: S100A4 and S100A6 proteins are well established markers of cell transformation and the S100B overexpression has been implicated in the Down's syndrome and Alzheimer's disease (Griffin et al. 1989). Differential expression of a panel of S100 proteins in various brain tumors may help in their diagnosis (Camby et al. 1999).

MECHANISM OF CELL SPECIFIC EXPRESSION OF THE S100 PROTEINS

Role of cis-acting promoter sequences and cell specific transcription factors

Studies on the regulation of expression of the S100 genes, especially these concentrated on the aspect of cell specific expression, are limited. In the case of some S100 genes there are indications that their expression only in certain cell types is based on the recognition of cis-acting elements in a gene promoter by cell specific transcription factors. The results come from studies carried out using DNA vectors comprising a reporter gene (encoding for example luciferase) under the control of a S100 gene regulatory region (promoter). After transfection into the cell, the relative activity of the reporter gene serves as an indicator of promoter efficiency. Comparing the results of transfection in cells that express a given protein, and these that do not, gives some indications as to the location of promoter regions acting as cell specific elements able to suppress or promote transcription upon interacting with cell specific transcription factors.

Results on the expression of S100D protein (calbindin 3), found in duodenal epithelial cells, may serve as an example of such mechanism. It was shown that a short promoter fragment (-117/+20) of the S100D gene generated protein expression in various types of cells, including these that did not express calbindin 3 under normal conditions (Lambert et al. 1996). However, longer promoter fragments revealed much lower activity in calbindin 3 negative HeLa cells than in epithelial cells that expressed calbindin 3. Moreover, a distant region of the gene (-4400/-2254) acted as a cell specific enhancer stimulating calbindin 3 expression only in duodenal epithelial cells. A cell specific Cdx-2 transcription factor binding in the TATA box region of the calbindin 3 promoter and present only in cells expressing calbindin 3 was identified. However, it was found that transfection of calbindin 3 negative cells with Cdx-2 expression vector was not sufficient to bring about calbindin 3 expression (Lambert et al. 1996).

A similar mechanism of expression may function for the S100B protein gene. Promoter fragments that may

act as cell specific repressors and inhibit transcription in cells other than glial cells were identified in murine (Jiang et al. 1993) and human (Castets et al. 1997) S100B gene, while a positive element enhancing transcription in glial cells was found within the rat S100B gene promoter (Hagiwara and Sueoka 1995). Studies of the S100A1 gene identified a distant regulatory region whose presence in the transfecting vector resulted in lower promoter activity in glial cells that exhibit very low levels of this protein but not in skeletal muscle cells which are abundant in S100A1 (Song and Zimmer 1996). In the case of the rat S100A4 gene, a 14 nucleotide long, GC rich sequence located 1300 bp downstream of the transcription start site was shown to diminish reporter gene expression in normal mammary epithelial cells characterized by low S100A4 expression (Chen et al. 1997) but not in metastatic cells abundant in S100A4. It was found that the transcriptional repression occurred due to the binding of a cell specific transcription factor, so called "GC factor"; the S100A4 expression was thus limited only to cells that did not express the GC factor.

Role of epigenetic factors in the control of cell specific expression of S100 genes

Calcyclin (S100A6) is present only in certain cell types (Kuźnicki et al. 1992, Timmons et al. 1993) and the molecular basis responsible for its limited expression is still unclear. Previous studies demonstrated that the human calcyclin gene promoter efficiency showed no apparent difference when transfected into melanoma cells with low and high calcyclin expression and nuclease sensitivity assay pointed for a different DNA accessibility in these cells (van Groningen et al. 1996). We have extended the studies to include four different cell lines as models for calcyclin positive and negative cells. Figure 1 shows that calcyclin mRNA was most abundant in Hep-2 human epithelial cells and also present in 10T1/2 mouse fibroblasts but absent in both C6 rat glioma and HepG-2 human hepatoma cells. These cells were transfected with calcyclin promoter/luciferase constructs in order to localize cis-acting elements within the calcyclin gene promoter, which might direct calcyclin expression in a cell specific manner. Figure 2A represents a schematic representation of the constructs used. As shown in Fig. 2B transfection with the promoterless construct resulted in no, or barely detectable, luciferase activity in cell extracts, while all types of cells transfected with plasmids containing the reporter gene under the control of calcyclin gene promoter fragments were able to express luciferase. These results suggest that, contrary to the data cited above on calbindin 3, S100B, S100A1 and rat S100A4 gene, within the calcyclin gene promoter, 3 kilobases 5' from the transcription start site, there is no sequence information that would drive transcription according to the physiological pattern, i.e. only in calcyclin-positive cells.

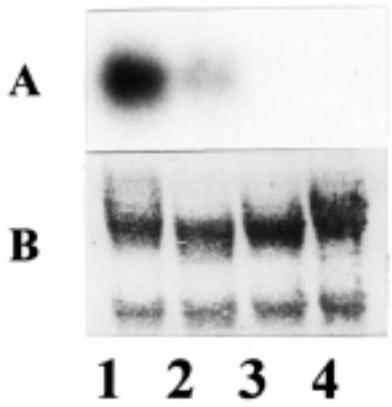

Fig. 1. Northern blot analysis of calcyclin mRNA in various cell lines. Ten μg of total RNA was loaded in each lane, electrophoresed, blotted on Hybond N-plus filters and immobilized by UV crosslinking. Calcyclin mRNA was probed with a 455 bp long calcyclin cDNA fragment labeled with [α-^{32}P]dCTP. Panel A; autoradiogram; Panel B; methylene blue staining of 28S and 18S RNA bands 1) Hep-2 human epithelial cells; 2) 10T1/2 mouse fibroblasts; 3) C6 rat glioma cells; 4) HepG-2 human hepatoma cells.

As the above studies did not identify a promoter region that would respond to cell specific transcription factors we extended our search to sequences contained in the first intron of the calcyclin gene. Two vectors were constructed that in addition to the -361/+134 calcyclin gene promoter fragment contained the first intron located either upstream or downstream of the promoter sequence (Fig. 2A). When these vectors were transfected into calcyclin positive human epithelial cells and calcyclin negative C6 rat glioma cells luciferase expression was lower as compared with the -361/+134 construct (Fig. 2C). The inhibitory effect was seen in both cell lines indicating that the presence of the intronic sequences does not result in cell specific repression of the calcyclin gene. Interestingly, such a pattern of ubiquitous expression from a promoter externally introduced into the cell is shared by at least two other S100 genes. The human S100A2 gene promoter was shown to be active, i.e. to drive the transcription of a reporter gene in all

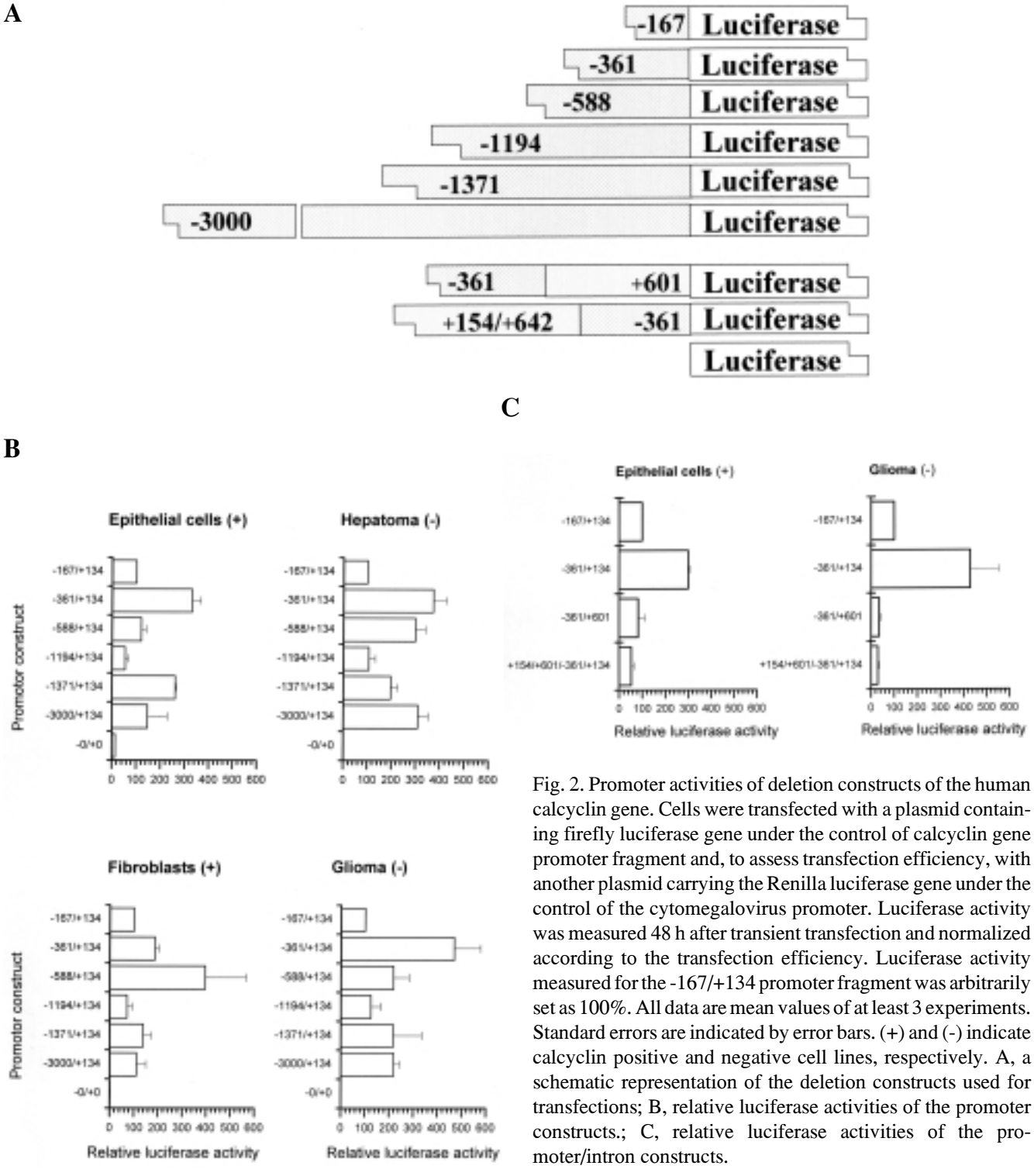

Fig. 2. Promoter activities of deletion constructs of the human calcyclin gene. Cells were transfected with a plasmid containing firefly luciferase gene under the control of calcyclin gene promoter fragment and, to assess transfection efficiency, with another plasmid carrying the Renilla luciferase gene under the control of the cytomegalovirus promoter. Luciferase activity was measured 48 h after transient transfection and normalized according to the transfection efficiency. Luciferase activity measured for the -167/+134 promoter fragment was arbitrarily set as 100%. All data are mean values of at least 3 experiments. Standard errors are indicated by error bars. (+) and (-) indicate calcyclin positive and negative cell lines, respectively. A, a schematic representation of the deletion constructs used for transfections; B, relative luciferase activities of the promoter constructs.; C, relative luciferase activities of the promoter/intron constructs.

cells studied regardless of whether the cells expressed the S100A2 or not (Wicky et al. 1997). Similar results were obtained for the mouse S100A4 gene promoter. In contrast to the data on the rat S100A4 gene promoter, none of the mouse S100A4 gene promoter fragments studied seemed to act in a cell specific way (Tulchinsky et al. 1992) and no repressory sequence, analogous to that found in the rat S100A4 gene promoter was found.

Since the promoters of the human calcyclin, human S100A2 and mouse S100A4 genes did not impose any cell specificity on the expression pattern of the respective genes, a different expression-controlling mechanism from that based on the interaction between promoter sequences and cell specific transcription factors should be considered. Indeed, it was shown that the expression of the S100A2 protein may be induced by treatment of the mammary carcinoma cells with 5-azadeoxycytidine, a well-established DNA demethylating agent, suggesting that gene methylation may contribute to the transcription control of the S100A2 gene (Lee et al. 1992). Further results confirmed the effect of 5-azadeoxycytidine on S100A2 expression and revealed a distinct methylation pattern in the S100A2 gene promoter in cells that expressed and did not express this protein (Wicki et al. 1997). Studies on the mouse S1004 gene revealed that the extent of methylation of cytosine residues in the first intron correlated with the absence of S100A4 expression (Tulchinsky et al. 1995). Latest results on the rat S100A4 gene also indicate that methylation of the first intron sequence contributes to the cell specific expression (Chen et al. 1999).

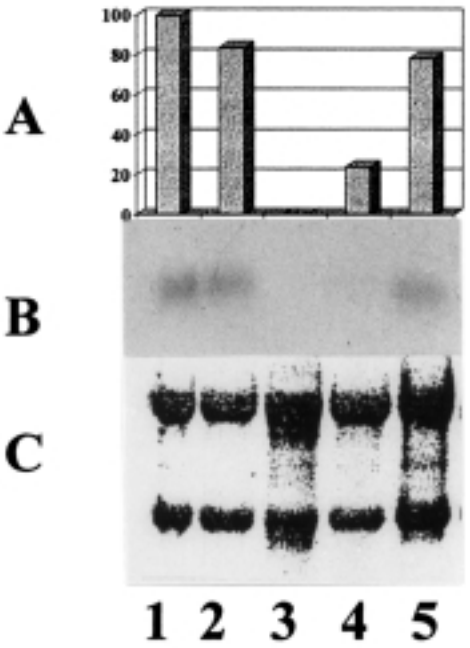

Fig. 3. Northern blot analysis of calcyclin mRNA in Hep-2 human epithelial and HepG-2 human hepatoma cells after treatment with 5-azacytidine. Ten μg of total RNA from control and 5-azacytidine treated cells was loaded in each lane, electrophoresed, blotted on Hybond N-plus filters and immobilized by UV crosslinking. Calcyclin mRNA was probed with a 455 bp long calcyclin cDNA fragment labeled with [α-^{32}P]dCTP. Hep-2 cells: 1) untreated; 2) treated with 4 μM 5-azacytidine for 6 days. HepG-2 cells: 3) untreated; 4) treated with 4 μM 5-azacytidine for 6 days; 5) treated with 10 μM 5-azacytidine for 12 days. Panel A, densitometric analysis; the signal from HepG-2 untreated cells was substracted from all other values; the signal from Hep-2 untreated cells was taken as 100%. Panel B, autoradiogram. Panel C, methylene blue staining of 28S and 18S RNA bands.

We have checked whether treatment of cells with 5-azacytydine may change the expression pattern of calcyclin. Calcyclin-positive epithelial cells and calcyclin-negative hepatoma cells were cultured in the presence of 4 μM 5-azacytidine for 6 days or of 10 μM 5-azacytidine for 12 days. The total RNA derived from these cells was examined for the presence of calcyclin mRNA by Northern blot analysis. As shown in Fig. 3, the amount of calcyclin mRNA does not change significantly in epithelial cells treated with 4 μM 5-azacytidine for 6 days. On the other hand, a radioactive band indicating the presence of calcyclin mRNA appears in total mRNA of hepatoma cells upon identical treatment while no calcyclin mRNA is detectable in untreated hepatoma cells. After 12 days in the presence of 10 μM 5-azacytidine the amount of calcyclin mRNA in hepatoma cells is similar to that in epithelial cells indicating that the action of 5-azacytidine can activate transcription from the calcyclin gene.

CONCLUDING REMARKS

The S100 calcium binding proteins are characteristic for their cell specific occurrence making them a good model to study the mechanism of cell specific expression. The review of current data available suggests that the regulation of the S100 protein expression, despite their homology and clustered localization of their genes, does not appear to be uniform. For some S100 proteins the pattern of their expression seems to depend on the presence of cell specific, cis-acting elements in their promoters which are functional, i.e., bring about protein expression only in certain cells that have the necessary transcription factors. There are, however, no common patterns and the regulation of each S100 gene seems to depend on the interaction of different cis-elements and transcription factors. On the other hand, the promoters of other S100 protein genes are functional in an indiscriminate fashion and the results indicate that cell specific ex-

pression may be determined by epigenetic factors, for example DNA methylation (Razin and Cedar 1991) or chromatin organization (Workman and Buchman 1993). The example of rat and mouse S100A4 gene suggests that the type of regulation may differ even between related species and that both gene methylation and the presence of cell specific cis-elements in the promoter may contribute to the overall pattern of expression.

The studies on S100A6 expression suggest that the promoter of its gene does not show any features determining cell specific expression and is thus functional in many cell types. The differences between the efficiency of the particular promoter fragments may reflect a modulatory influence on the transcription rate rather than control of the on/off switch of transcription. No cell specific features seem to be present in the sequence of the first intron of the calcyclin gene, either. Our preliminary results on the effect of a DNA demethylating agent on calcyclin expression and earlier observation that the chromatin region comprising the calcyclin gene could have a different nucleosome arrangement in cells with high and low calcyclin level (van Groningen et al. 1995) suggest an epigenetic control of calcyclin gene expression.

Further studies determining the actual methylation state of the calcyclin gene in various cell types and mapping the respective methylation sites are necessary. The studies on factors initiating the changes in calcyclin gene accesibility and leading to the upregulation of its expression in some pathological states would be of special interest.

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