Antisense Inhibition of Glial S100β Production Results in Alterations in Cell Morphology, Cytoskeletal Organization, and Cell Proliferation

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Abstract. The phenotypic effects of selectively decreasing the levels of S100β in cultured glial cells were analyzed. Two separate antisense approaches were utilized for inhibition of S100β production: analysis of clonal isolates of rat C6 glioma cells containing an S100β antisense gene under the control of a dexamethasone-inducible promoter, and analysis of C6 cells treated with S100β antisense oligodeoxynucleotides. Both antisense methods resulted in a decrease in S100β levels in the cell, as measured by RIA. The inhibition of S100β production correlated with three alterations in cellular phenotype: (*a*) a flattened cell morphology; (*b*) a more organized microfilament network; and (*c*) a decrease in cell growth rate. The studies described here provide direct evidence for an involvement of S100β in glial cell structure and function, and suggest potential in vivo roles for S100β in regulation of glial cell morphology, cytoskeletal organization, and cell proliferation.

S100β is a calcium binding protein that is synthesized in glial cells of vertebrate brain, and that is highly conserved in amino acid sequence among vertebrate species (see Donato, 1986; Van Eldik and Zimmer, 1988 for reviews). S100β is also a member of a larger class of small, acidic proteins with extensive amino acid sequence similarities. This family of proteins (some of whose sequences have been inferred from cDNA sequences) includes calcium binding proteins, proteins that increase in cells after growth factor or serum stimulation, proteins that increase upon differentiation or transformation, a protein subunit of a tyrosine kinase substrate, and proteins that are found in diseases like cystic fibrosis and rheumatoid arthritis (see Winningham-Major et al., 1989 for references). The observation that expression of many of these proteins is altered during cell growth or differentiation suggests that they may be involved in regulation of these processes. However, little information is available about the in vivo roles of any of these proteins, including S100β.

The biochemistry, localization, and in vitro activities of S100β have been studied extensively over the past 20 years, and the immunochemical detection of S100β is a standard procedure in diagnostic pathology. Based mostly on the results of in vitro reconstitution studies, several roles for S100β in glial cell function have been suggested. S100β has been reported to alter protein phosphorylation, microtubule disassembly, and an intracellular enzyme (see Van Eldik and Zimmer, 1988 for references), suggesting that S100β may have multiple regulatory activities in glial cells. In addition to intracellular functions, there is evidence that some forms of S100β may be secreted and function in an extracellular role in nervous tissue. For example, S100β has been detected in brain extracellular fluid (Shashoua et al., 1984) and in conditioned media from glial cells (Suzuki et al., 1987; Van Eldik and Zimmer, 1987), and a disulfide-linked form of S100β has neurotrophic activity; i.e., it can stimulate neurite outgrowth (Kligman and Marshak, 1985; Kligman and Hsieh, 1987; Van Eldik et al., 1988; Winningham-Major et al., 1989) and enhance cell survival (Winningham-Major et al., 1989) in cultures of CNS neurons.

S100β protein levels appear to be subject to regulation. For example, S100β levels can be increased in cell lines (Tsunamoto et al., 1988; Van Eldik and Zimmer, 1987; Zimmer and Van Eldik, 1989) and in response to activation of the cAMP signal transduction pathway (Labourdette and Mandel, 1980; Higashida et al., 1985; Zimmer and Van Eldik, 1989). In addition, the gene for human S100β has been mapped to the distal arm of chromosome 21 in the Down's Syndrome region (Allore et al., 1988; Duncan et al., 1989), and S100β levels have been reported (Griffin et al., 1989) to be increased in reactive glial cells of patients with Down's syndrome and Alzheimer's disease. The results from our laboratory and others clearly raise the possibility that S100β may play critical in vivo roles that affect both the glial cell where it is synthesized, and the neuronal cell, a possible target of secreted S100β. Based on this model of S100β action on both glial and neuronal cells, abnormalities of S100β gene expression and protein production/targeting could have pro-

found effects on nervous system function. However, the mechanistic relationship of altered S100β levels to cellular abnormalities is not known, and there has not been a direct analysis of how perturbation of S100β levels in a selective and specific manner correlates with changes in cell phenotype. The development of a biological system where the S100β levels can be altered (either increased or decreased) in a selective and reproducible manner might provide a useful system for examination of the role of S100β in glial cell structure and function.

In the studies reported here, we have analyzed the effects of a selective decrease in S100β levels in rat C6 glioma cells. C6 cells have been used for many years as a model glial cell system (Benda et al., 1968), and the localization and levels of S100β in C6 cells have been extensively studied (see Van Eldik and Zimmer, 1988 for references). We have previously reported that C6 cells contain predominantly, if not exclusively, the β subtype of S100 (Zimmer and Van Eldik, 1988), and that S100β can be released into the conditioned medium (Van Eldik and Zimmer, 1987). Thus, the C6 cell provides an excellent biological system amenable to experimental manipulations, and useful for studying how inhibition of S100β production affects cellular phenotype.

We report here studies using two "antisense" approaches for inhibiting S100β production in C6 cells: (*a*) characterization of C6 clones containing an S100β antisense gene construct under the control of a dexamethasone-inducible promoter, and (*b*) analysis of C6 cells treated with S100β antisense oligonucleotides. Our initial data analyzing the phenotypic consequences of selectively decreasing S100β levels suggest important roles for S100β in glial cell morphology, cytoskeletal organization, and growth regulation.

Materials and Methods

Cell Culture

Rat C6 glioma cells were obtained from American Type Culture Collection (Rockville, MD) and grown in α-MEM (Gibco Laboratories, Grand Island, NY) supplemented with 2.5% (vol/vol) FBS (Hyclone Laboratories; Logan, UT), 40 U/ml penicillin, and 40 μg/ml streptomycin (Gibco Laboratories). The cells were grown at 37°C in a humidified atmosphere containing 7% CO_2. C6 cells that contained the pS100AS plasmid were grown in α-MEM with FBS that had been treated with charcoal to deplete steroids. Steroids were depleted from the FBS by adding activated charcoal (10 g/500 ml serum) and heating at 55°C for 30 min while stirring. The serum/charcoal mixture was then centrifuged at 35,000 *g* for 45 min at 4°C, and the supernatant was sterilized by filtration through 0.22-μm filters.

Preparation of S100β Antisense Vector

A plasmid (pS100AS) containing the S100β antisense gene was constructed by using a synthetic S100β gene (Van Eldik et al., 1988). Briefly, the S100β coding region was isolated from pVUSB-1 (Van Eldik et al., 1988) by treatment with Eco RI and Hind III. The 289-bp insert was purified by electroelution, treated with T4 polymerase to generate blunt ends, and ligated into the Eco RV site in the polylinker region of the dexamethasone-inducible, pMSVneo eukaryotic expression vector (Chung et al., 1988; obtained from Dr. Claire Fraser, National Institutes of Health).

Ligation, transformation and selection of recombinant plasmids in *Escherichia coli* were done as previously described (Van Eldik et al., 1988) and orientation of the S100β insert was determined by restriction enzyme mapping with Sac I, Xba I, and Nru I. The vector containing the S100β gene in the antisense orientation was termed pS100AS.

Isolation of C6 Clones Containing pS100AS

C6 cells were cotransfected with pS100AS (10 μg) and a plasmid (pRShGRa; 10 μg) containing a glucocorticoid receptor insert (Giguere et al., 1986). Transfections were done by a calcium phosphate precipitation technique (Wigler et al., 1978) and glycerol shock (Mellon et al., 1981). Cells that stably integrated pS100AS were selected by their ability to grow in 400 μg/ml geneticin (G418; Sigma Chemical Co.). Colonies of cells were subcloned using cloning rings and expanded in the selection medium. C6 clones that contained the S100β antisense gene construct were designated C6-AS1, C6-AS2, etc.

To induce expression of the S100β antisense gene, cells were treated for various times with 1 μM dexamethasone (10 μl of 1 mM dexamethasone in ethanol added per 10 ml medium). Controls received an equivalent concentration of diluent alone (10 μl ethanol per 10 ml medium). Experiments were designed so that at each time point, cells were harvested at similar cell densities (80% confluency).

Analysis of Morphological Changes

Morphological examination of cells containing the pS100AS plasmid was made by video-enhanced differential interference contrast microscopy, following the methods described by Schnapp (1986). Cells were placed on glass coverslips at a density of 1×10^4 cells/ml, and grown for 24 h in the presence or absence of 1 μM dexamethasone (Sigma Chemical Co., St. Louis, MO). Coverslips were then placed cell side down onto glass microscope slides and the edges sealed with a mixture of melted lanolin, vaseline, and paraffin (1:1:1). Slides were then inverted and placed on a Zeiss Axiovert 35 microscope mounted inside a 37°C air bath as described by Hitt et al. (1990). Interference contrast images were made using a 100× Neofluor objective, 2.5× Optovar lens, and Dage Model 67 video camera. Photographs were taken directly from the video monitor.

The cytoskeletal proteins actin, tubulin, and vimentin were localized by fluorescence methods. For these experiments, cells were grown on glass coverslips in the presence or absence of 1 μM dexamethasone for 72 h, fixed in 3.7% formaldehyde for 30 min at 22°C, and permeabilized in −20°C acetone for 7 min (Welsh, 1983). Actin distribution was visualized using phallacidin (Bodipy-phallicidin; Molecular Probes, Inc., Eugene, OR) as an affinity probe (Barak and Yocum, 1981). Tubulin and vimentin distributions were analyzed by immunofluorescence as previously described (Welsh, 1983) using mouse mAbs TUB 2.1 and VIM-13.2 (Sigma Chemical Co.), respectively.

Analysis of S100β Levels in Cell Extracts

S100β protein levels were determined by RIA of cell extracts enriched for S100β. Cells were harvested at 80% confluency by rinsing the plates three times with PBS, scraping the cells into PBS, and centrifuging at 3,000 *g* for 5 min at 4°C to collect the cell pellet. Cell pellets were resuspended in homogenization buffer (1 vol buffer/vol pellet) containing 50 mM Tris-HCl, 1 mM EGTA, 1 mM β-mercaptoethanol, pH 7.4; and homogenized by sonication (three times for 30 s each) using a microprobe on a dismembrator (model 300; Fisher Scientific, Fair Lawn, NJ) at 30% relative energy output. The homogenate was centrifuged at 16,000 *g* for 5 min at 4°C, the resultant supernatant was placed in a boiling water bath for 7 min, and the heat-treated supernatant was centrifuged again at 16,000 *g* for 5 min. The pH of the final supernatant was lowered to 4.0 by the addition of 1 M H_2SO_4, and the mixture incubated for 2 h at 4°C. Precipitated proteins were then collected by centrifugation at 3,000 *g* for 5 min at 4°C. The pellet was resuspended in RIA buffer (20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.6), and the pH of the solution was raised to 7.5 by addition of 0.1 N NaOH. The solution was incubated overnight at 4°C and the pH was then readjusted with NaOH to a final pH of 7.5. S100β levels in these partially purified samples were measured by RIA as previously described (Zimmer and Van Eldik, 1988).

Protein determinations were done by the method of Lowry et al. (1951) using BSA as a standard.

Synthesis and Purification of Oligonucleotides

Oligodeoxynucleotides were synthesized on an automated solid-phase synthesizer (model 380B; Applied Biosystems, Inc., Foster City, CA) by β-cyanoethyl phosphoramidite chemistry. Crude, detritylated oligonucleotides were dissolved in 100 μl sterile water or PBS and further purified using Sephadex G-25 spin columns. Spin columns were constructed with a 1-ml tuberculin syringe containing sterile siliconized glass wool and filled to the 0.8-ml level with preswollen, sterilized Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, NJ). Spin columns were washed with 4 ml of sterile

water or PBS by centrifugation at 1,500 *g* for 3 min at 4°C. The oligonucleotide was added to the resin bed and eluted by centrifugation of the column at 1,500 *g* for 10 min at 4°C into a sterile tube. Final oligonucleotide concentration was then determined by spectrophotometry.

Treatment of C6 Cells with Oligonucleotides

All studies for determining the effects of S100β antisense oligonucleotides on C6 cells used early passage cells (<14 passages after obtaining cells from American Type Culture Collection). Cells were plated at a density of 0.5×10^4 cells/ml in 24-well tissue culture plates and grown for 24 h in α-MEM containing 2.5% FBS. The serum-containing medium was then aspirated from the cells, and the cells were washed two times in serum-free α-MEM. Cultures were then incubated in serum-free α-MEM containing 30 μM oligonucleotides. After a 2-h incubation, FBS was added to the wells (without changing the medium) to a final serum concentration of 2.5%. Preliminary experiments were carried out essentially as described (Holt et al., 1988) to demonstrate that the FBS serum lot (lot 1115864; Hyclone Laboratories) had low nuclease activity (data not shown). Cells were incubated in the presence of oligonucleotides for various times, and cell counts were determined with a hemacytometer at 24-h intervals. In experiments examining the effect of oligonucleotides on S100β levels, experiments were designed so that at each time point, cells were harvested at similar cell densities (80% confluency). For the studies reported here, cells received only a single addition of oligonucleotide at the beginning of the time course, and there was no medium change during the experiment.

Results

Isolation of C6 Cells Containing an S100β Antisense Gene

A eukaryotic expression vector capable of producing RNA that is complementary to the coding sequence of an S100β gene was constructed; key features of this S100β antisense vector are diagrammatically summarized in Fig. 1. As described in Materials and Methods, the S100β coding region was isolated from pVUSB-1, a vector containing a synthetic S100β gene (Van Eldik et al., 1988), and then inserted into the polylinker region of the pMSVneo eukaryotic expression vector (Chung et al., 1988). The pMSVneo vector was chosen for several reasons. First, it allows the inducible expression of eukaryotic genes in mammalian cells. The presence of sequences in the long terminal repeat of the mouse mammary tumor virus allows regulation of expression by addition of glucocorticoids to the culture medium. This is advantageous because clones can be selected in the absence of inducer and the effects of gene induction can be followed in the same cells. Second, pMSVneo contains the neomycin resistance gene, allowing selection of stably transformed cells when grown in medium containing the neomycin analogue, Geneticin (G418). Third, pMSVneo contains a polylinker region with several unique restriction enzyme sites for easy insertion of foreign DNA. Fourth, pMSVneo contains an ampicillin resistance gene and pBR322 origin or replication, allowing initial cloning in bacterial cells.

Orientation of the S100β gene insert was determined by restriction enzyme mapping, and the vector containing the gene in the antisense orientation was designated pS100AS (Fig. 1). C6 cells were transfected as described in Materials and Methods, and stable transformants were selected and expanded under G418 selection. A number of C6-derived clones were found to be resistant to G418, and these clones have been termed C6-AS1, C6-AS2, et cetera. As discussed below, we examined selected clones for steroid-inducible changes.

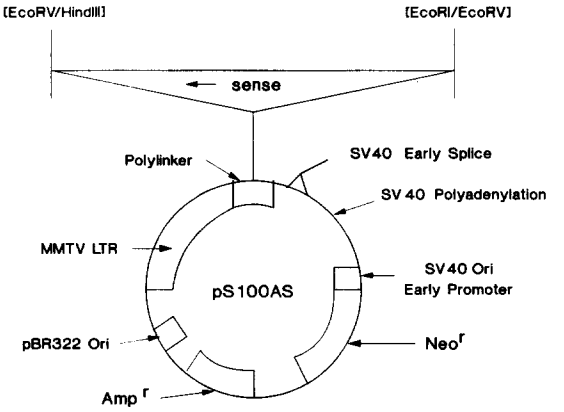

Figure 1. S100β antisense vector. This diagram, while not to scale, shows the key features of the eukaryotic expression vector (pS100AS) containing an S100β gene in the antisense orientation. A 279-bp fragment containing the entire coding region of a synthetic S100β gene was isolated from pVUSB-1 (Van Eldik et al., 1988) by digestion with Eco RI and Hind III, blunt-ended, and inserted into the Eco RV site of the polylinker region of pMSVneo (Chung et al., 1988). Cloning in *E. coli* used the ampicillin resistance gene (*Amp^r*) and the pBR322 origin of replication (pBR322-ori). After transfection of C6 glioma cells, the neomycin resistance gene (*Neo^r*) expressed from an SV40 origin and early promoter allowed selection of positive clones in G418. The dexamethasone-inducible promoter in the mouse mammary tumor virus–long terminal repeat (MMTV LTR), and the SV-40 early splice and polyadenylation sequences are also shown.

Changes in Cell Morphology and Cytoskeletal Organization in C6-AS Clones

Upon addition of dexamethasone to induce expression of the S100β antisense gene, we observed dramatic changes in cell morphology in several of the clones. An example of a representative clone (C6-AS1) showing the morphological changes is depicted in Fig. 2. In the absence of dexamethasone, the cells showed the typical bipolar, stellate shape similar to the parental, untransfected C6 cells. However, after dexamethasone addition, the majority of cells exhibited a flattened, "fried egg-like" appearance. Quantitation of the relative areas of a representative population of cells by using Bioquant Elite Software (R&M Biometrics, Nashville, TN) demonstrated that there was a threefold increase in area after dexamethasone treatment. The morphological change does not appear to be a nonspecific dexamethasone effect, since the changes were not seen in the untransfected C6 cells treated with dexamethasone. Although the time course of the morphological changes varied with the particular clone, in general the changes were gradual, with a small proportion of the cells in a culture showing the flattened, enlarged morphology at 24 h after dexamethasone addition, and the majority of cells affected by 72 h after dexamethasone addition.

Dexamethasone treatment of C6-AS clones also resulted in alterations in cytoskeletal organization, as assayed by fluorescence localization of cytoskeletal proteins. These results are shown in Fig. 3. Analysis of microfilaments by staining with the actin-selective stain, phallacidin, showed differences in actin distribution in C6-AS1 cells grown in the absence (Fig. 3 *A*) or presence (Fig. 3 *B*) of dexamethasone for 72 h. After treatment with dexamethasone, the C6-AS1

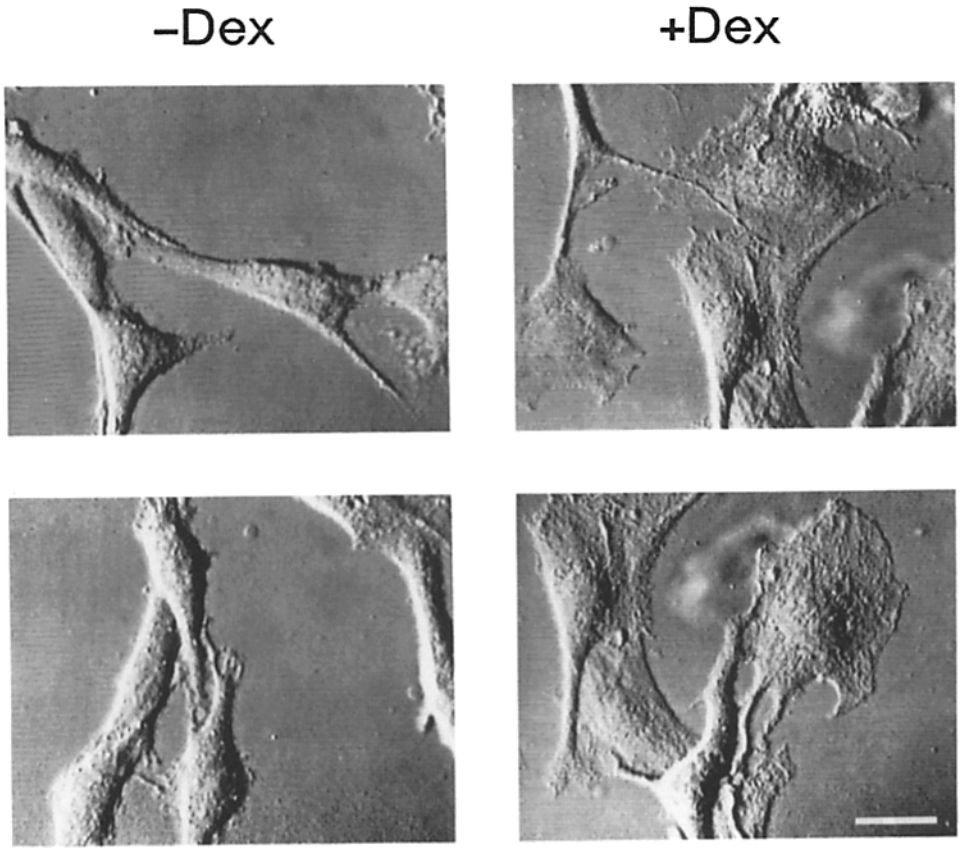

Figure 2. Effects of S100β antisense gene induction on cellular morphology. A C6 clone (*C6-AS1*) containing the S100β antisense gene under the control of a dexamethasone-inducible promoter was grown for 24 h in the absence (*left*) or presence (*right*) of 1 μM dexamethasone. Photographs were made from a video screen display of cells viewed under Nomarski optics as described in Materials and Methods. Bar, 1 μm.

cells showed a more organized actin stress fiber staining pattern with flatter, even arrays of microfilament bundles close to the culture substrate. The cells also appeared to show less membrane ruffling. In contrast to the results with actin staining, analysis of intermediate filaments and microtubules by staining with antibodies to vimentin (Fig. 3, *C* and *D*) and tubulin (Fig. 3, *E* and *F*), respectively, showed no obvious changes in the dexamethasone-treated cells. These initial data indicate that induction of the S100β antisense construct with dexamethasone results in a flattened cell morphology and changes in the distribution of the microfilament network of the cellular cytoskeleton.

Decreased Levels of S100β in the C6-AS Clones

To determine if the morphological effects seen in C6-AS clones correlated with an inhibition of S100β production, we measured S100β levels in the cells by radioimmunoassay. Three clones were selected for analysis of S100β levels before and after induction of antisense expression by dexamethasone: two clones (C6-AS1 and C6-AS2) that showed the altered morphology in response to dexamethasone, and one clone (C6-AS3) which showed no obvious morphological change in response to dexamethasone. The parental, untransfected C6 cells were also examined. As discussed in Materials and Methods, cells were treated with dexamethasone for different lengths of time, and S100β levels in partially purified cell extracts were determined by RIA. Because the total levels of S100β varied with the particular clone examined, the data for each clone are expressed as a percentage of the S100β levels at the 0-h time point (i.e., in the absence of dexamethasone). The results for one of the clones (C6-AS2) are shown in Fig. 4.

As can be seen in Fig. 4 *A*, the addition of dexamethasone to the C6-AS2 cells caused a significant, time-dependent decrease in S100β levels below control levels (S100β levels in the absence of dexamethasone). Specifically, the S100β levels were only 40% of control at 48 h after dexamethasone; by 96 h of dexamethasone treatment, the S100β levels were only 16% of control levels. The C6-AS1 cells showed a similar pattern of inhibition of S100β levels after dexamethasone treatment (data not shown). In contrast, the addition of dexamethasone to the parental, untransfected C6 cells did not inhibit the levels of S100β (Fig. 4 *B*). Finally, a clone (C6-AS3) that did not show the altered morphology in the presence of dexamethasone also did not show a dexamethasone-induced decrease in S100β levels (data not shown). Altogether, these data are consistent with the hypothesis that the morphological and cytoskeletal changes we observe after dexamethasone treatment are a result of a decrease in S100β levels in the cells.

Effects of S100β Inhibition on Cellular Proliferation

Induction of the S100β antisense gene with dexamethasone resulted in a decrease in cellular growth rate. For example, in one experiment, the addition of dexamethasone to the C6-AS1 clone resulted in fewer than half the number of cells in the plate after 3 d compared with cells without dexamethasone. However, we found that dexamethasone also inhibited the growth rate of the parental, untransfected C6 cells. Thus, we could not distinguish between an effect of dexamethasone

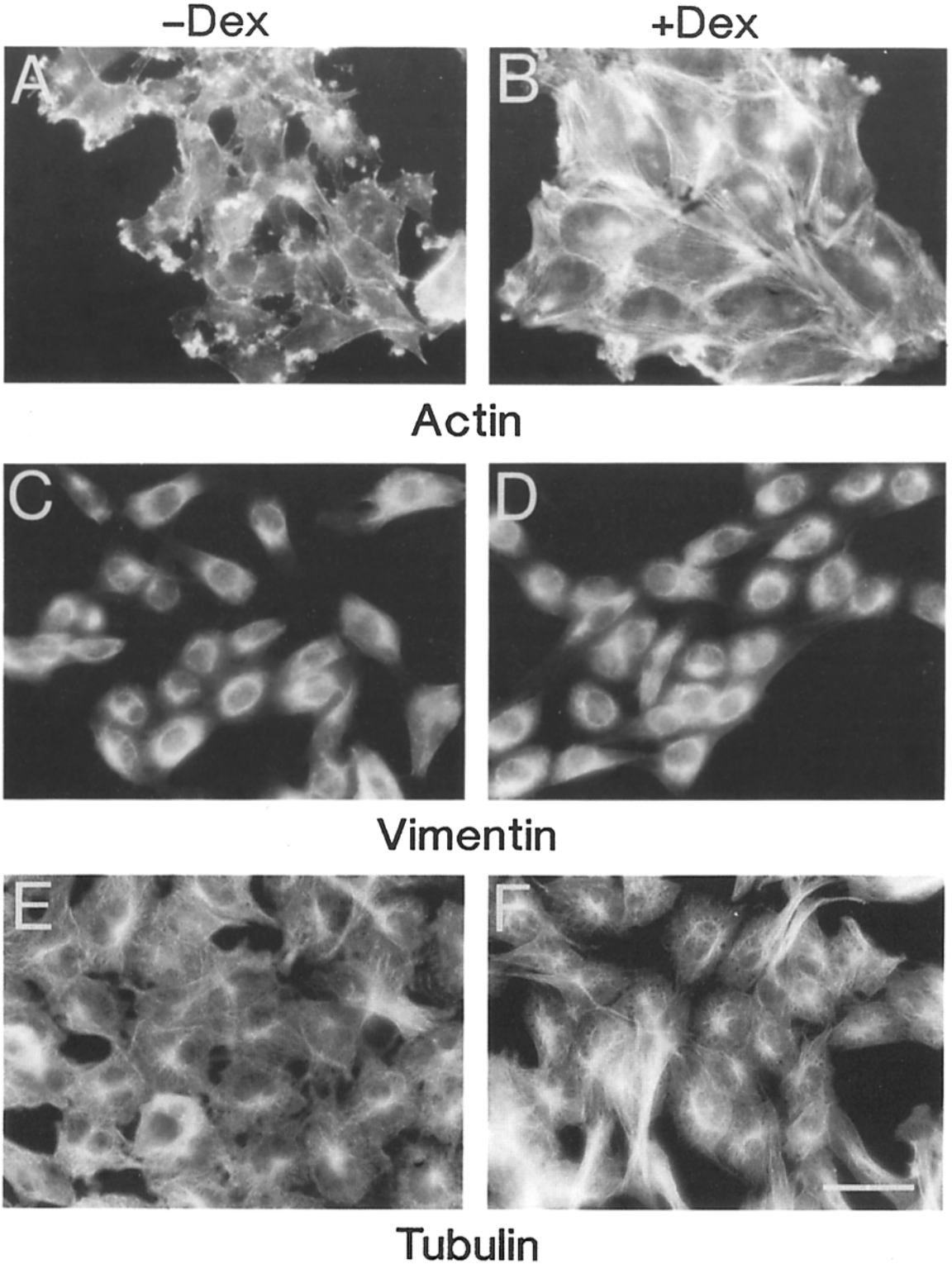

Figure 3. Effects of S100β antisense gene induction on cytoskeletal structure. A C6 clone (*C6-AS1*) was grown for 72 h in the absence (*A*, *C*, and *E*) or presence (*B*, *D*, and *F*) of dexamethasone. Fluorescence analysis of actin (*A* and *B*), and immunofluorescence analysis of vimentin (*C* and *D*), and tubulin (*E* and *F*) distributions were done as described in Materials and Methods. Bar, 50 μm.

alone on growth vs. an effect of S100β inhibition on growth. To address specifically the effect of decreased S100β levels on cell proliferation, we used another method for S100β inhibition, the use of S100β antisense oligonucleotides.

We prepared an S100β antisense oligonucleotide whose sequence is the inverse complement of 15 bases of the rat S100β cDNA sequence (Kuwano et al., 1984), beginning at the ATG codon that corresponds to the initiator methionine.

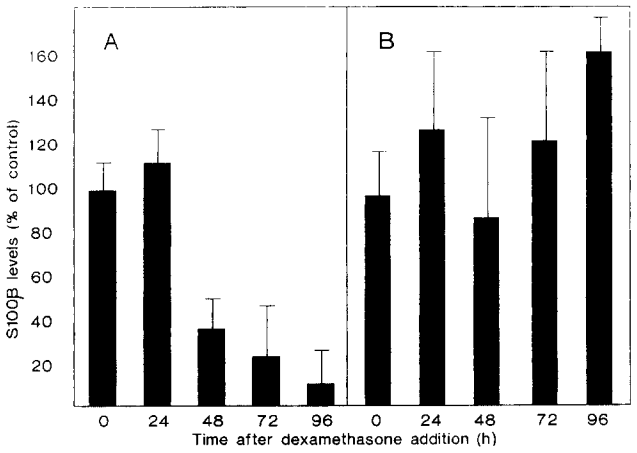

Figure 4. Inhibition of S100β production in a C6 clone containing the S100β antisense gene. The C6-AS2 clone (*A*) and untransfected C6 cells (*B*) were grown in the presence of dexamethasone for various lengths of time. S100β levels in cell extracts were determined by RIA, and calculated as nanograms S100β/micrograms total protein. S100β levels are expressed as a percentage of the control levels at the 0-h time point (i.e., in the absence of dexamethasone). Bars represent the mean ± SEM of four determinations (two separate experiments analyzing two wells per time point).

As controls, we synthesized the sense oligonucleotide that is equivalent to the coding strand of the cDNA in this region, and a mismatch oligonucleotide that is equivalent to the antisense sequence in which two bases have been changed. A search of sequence databases revealed no matches (other than to S100β) of the oligonucleotides with any known nucleotide sequence. The sequences of the oligonucleotides used in this study are shown in Fig. 5.

We then tested the effects of addition of the oligonucleotides to cultures of untransfected C6 cells. We found that C6 cells treated with the S100β antisense oligonucleotide showed morphological changes similar to the stable transformants treated with dexamethasone, i.e., they exhibited a flattened and more spread appearance, with a 4.4-fold increase in cellular area. No such changes in morphology were observed in C6 cells treated with the sense or mismatch oligonucleotides. In addition, as shown in Fig. 6, treatment of C6 cells with the antisense oligonucleotide resulted in a time-dependent decrease in cellular growth rate, as assayed by cell number, when compared with cells grown in the presence of the sense oligonucleotide or in the absence of oligonucleotide. A significant decrease in cell number was seen by 48 h after the addition of a single dose (30 μM) of antisense oligonucleotide. In other experiments, we have seen inhibition of cell growth rate with lower doses of antisense oligonucleotide. We did not see similar inhibition of proliferation when the sense or mismatch oligonucleotides were tested.

We confirmed by RIA that treatment of C6 cells with the antisense oligonucleotide resulted in a decrease in intracellular S100β levels compared with the S100β levels in cells treated with sense oligonucleotide or untreated cells. As shown in Fig. 7, in cells treated with antisense oligonucleotide for 72 h, the S100β levels were ~20% that of the untreated cells or cells treated with sense oligonucleotide. Altogether, these data suggest that a selective inhibition of S100β production in C6 cells results in a decreased rate of cellular proliferation, and implicate S100β as a component integral to glial cell growth.

Discussion

We have demonstrated here that a selective inhibition of S100β production in glial cells is correlated with at least three phenotypic changes: (*a*) a more flattened cellular morphology; (*b*) an alteration in cytoskeletal organization at the level of the microfilament network; and (*c*) a decrease in cellular growth rate. Similar results obtained with two distinct antisense methods support the conclusion that S100β has important roles in regulation of glial cell morphology, cytoskeletal structure, and cell proliferation.

Antisense approaches are increasingly being used with a variety of eukaryotic genes in attempts to understand the function of specific gene products by inhibiting the production of the protein and analyzing the functional consequences (see Green et al., 1986 for review). The S100β gene (Van Eldik et al., 1988) used in our studies has a 77% homology with the coding region of rat S100β cDNA (Kuwano et al., 1984). Previous studies (Holt et al., 1986) showed that complete homology is not required for inhibition of gene function by antisense constructs, and we confirmed here by RIA that the S100β levels in the cells were decreased after induction of the antisense gene construct with dexamethasone. The use of an antisense gene under the control of an inducible promoter was also advantageous because antisense-containing clones could be selected before S100β inhibition occurred, thus avoiding potential problems in cloning due to decreases in cell growth rate.

The use of antisense approaches has allowed us to begin to address the in vivo roles of S100β in glial cells. Our data suggest that S100β is involved in regulation of cell morphology, cytoskeletal structure, and cell proliferation. The observation of a more flattened cellular appearance after inhibition of S100β production in C6 cells is consistent with the effects seen on microfilament organization, i.e., the flatter, more organized actin stress fiber staining pattern and decreases in membrane ruffling. These alterations in microfila-

Sense	5' ATG TCT GAG CTG GAG 3'
Antisense	3' TAC AGA CTC GAC CTC 5'
Mismatch	3' TAG AGA CTC CAC CTC 5'

Figure 5. Sequences of S100β oligonucleotides. The sense oligonucleotide is equivalent to 15 bases of the rat S100β cDNA sequence (Kuwano et al., 1984), beginning at the ATG codon corresponding to the initiator methionine. The antisense oligonucleotide is the inverse complement of the sense strand sequence. The mismatch oligonucleotide is equivalent to the antisense oligonucleotide sequence in which two bases have been changed. The sequences of the antisense and mismatch oligonucleotides are depicted in the 3' to 5' orientation for ease of comparison with the sense oligonucleotide. The rat S100β cDNA sequence is available from EMBL/GenBank/DDBJ under accession number X01090.

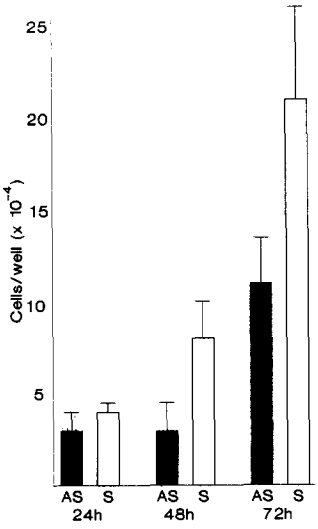

Figure 6. Effect of S100β antisense oligonucleotides on cell proliferation. As described in Materials and Methods, C6 cells were incubated in the presence of 30 μM antisense oligonucleotide (AS; *solid bars*) or sense oligonucleotide (S; *open bars*) for various lengths of time, and cell numbers were determined. Bars represent the mean ± range of duplicate cultures from a representative experiment.

ment organization were somewhat unexpected, however. Previous in vitro studies have suggested that S100β can affect the microtubule network. For example, in reconstitution studies, S100β has been reported to stimulate microtubule disassembly and to interact *in vitro* with tubulin or other microtubule-associated proteins (Baudier et al., 1982; Endo and Hidaka, 1983; Baudier and Cole, 1988; Donato et al., 1989; Donato and Giambanco, 1989, and references therein). However, under the conditions used in the in vivo study reported here, a decrease in S100β levels in C6 glioma cells did not appear to affect the microtubule network. Instead, we detected alterations in the microfilament network of the cellular cytoskeleton.

Our studies demonstrate the need for a detailed in vitro analysis of S100β effects on microfilaments. These kinds of studies might provide insight into molecular mechanisms involved in the effects we observed. For example, these studies might elucidate whether S100β has a direct effect on components of the microfilament structure (such as actin or actin-binding proteins) or whether the effects are more indirect (such as through protein phosphorylation/dephosphorylation mechanisms). Our results are consistent with a model whereby S100β may affect both microfilaments and microtubules, and that the balance of cytoskeletal structure and dynamics may be related to the levels and/or localization of S100β. The availability of glial cell lines amenable to experimental manipulation and that show selective decreases in S100β levels provides a potential starting point for directly testing these possibilities.

In the studies reported here, we also showed that inhibition of S100β production in C6 cells is correlated with an inhibition of cellular proliferation rate. This result was not unexpected, since a potential role for S100β in cellular growth processes is suggested by its sequence similarity to a number of proteins that appear to be growth related; i.e., their expression correlates with cell cycle or cell growth parameters. Our results suggest that the levels or concentration of S100β in the glial cell is related to the rate of cellular proliferation. At this time, it is unclear whether S100β is affecting cell growth as an essential compound (i.e., necessary but not sufficient) or as an active, regulatory component used by the cell to control its proliferation. Previous evidence has implicated S100 in stimulation of [^{3}H]thymidine incorporation in certain cells (Klein et al., 1989) or in regulation of progression through the cell cycle (Marks et al., 1990). However, a direct demonstration that increases in S100β levels correlate with increased cell growth or that S100β actively stimulates increases in glial cell number has not yet been reported. Studies examining a potential regulatory role for S100β in C6 cell proliferation are in progress.

Interestingly, the cellular parameters affected by reduction of S100β levels are also affected by the process of cellular transformation. The effects of decreased S100β levels on cell morphology and growth rate are consistent with a reversal of the transformed phenotype of the parental C6 glioma cell line. Microfilament stress fibers appear to be indicators of the dependence of normal cells on anchorage to a substratum, and transformation of many cell types by viral infection or oncogene activation results in the loss of this flattened morphology and the acquisition of the rounded appearance common to C6 cells (for review, see Burridge, 1986). Relatedly, the process of transformation can often increase the growth rate of a cell population in low concentrations of serum or growth factors (Holley, 1975). As inhibition of S100β production in the transformed C6 cells resulted in a phenotype more characteristic of normal cells, it is interesting to speculate that S100β may play an important role in mediation of some aspects of transformation. In this respect, it will be important to determine the effects of S100β on other parameters of transformation such as anchorage-independent growth, foci formation, and activities of certain growth-related kinases.

The results reported here, taken together with previous studies, suggest that homeostasis of S100β levels in the glial cell may be important for normal glial and neuronal function. While the mechanistic relationship of aberrant produc-

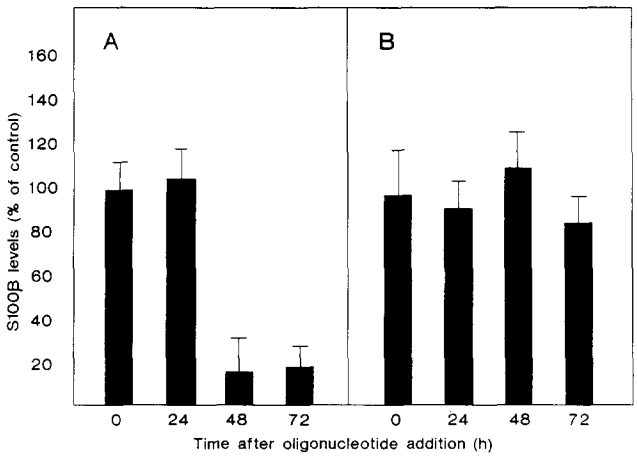

Figure 7. Inhibition of S100β production in C6 cells treated with antisense oligonucleotides. C6 cells were treated with 32 μM antisense oligonucleotide (*A*) or sense oligonucleotide (*B*) for various lengths of time. S100β levels in cell extracts were determined by RIA, and calculated as nanograms S100β/micrograms total protein. S100β levels are expressed as a percentage of the control levels (the levels in untreated C6 cells at each time point). Bars represent the mean ± range of duplicate cultures from a representative experiment.

tion of S100β to cellular abnormalities is not known, several observations are consistent with the hypothesis that abnormal regulation of S100β levels may lead to specific neuropathologies: (*a*) a form of S100β has neurotrophic activity in CNS; (*b*) the human S100β gene is localized to the Down syndrome region of chromosome 21, raising speculation that S100β may be involved in neurologic deficits associated with Down's syndrome or Alzheimer's disease; (*c*) S100β interacts in vitro with a microtubule-associated protein that is a component of neurofibrillary tangles; and (*d*) the data presented here suggest an important role for S100β in cellular structure and proliferation. The studies reported here have provided new perspectives on possible in vivo roles of S100β in glial cell morphology, cytoskeletal organization, and growth regulation. In addition, these data provide a framework for future studies aimed at defining the molecular mechanisms by which S100β is involved in nervous system development and maintenance.

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References

Allore, R., D. O'Hanlon, R. Price, K. Neilson, H. F. Willard, D. R. Cox, A. Marks, and R. J. Dunn. 1988. Gene encoding the β subunit of S100 protein is on chromosome 21: implications for Down syndrome. *Science (Wash. DC).* 239:1311–1313.

Barak, L. S., and R. Yocum. 1981. Nitrobenz-2-oxa-1,3-diazole (NBD)-phallacidin: synthesis of a fluorescent actin probe. *Anal. Biochem.* 110: 31–38.

Baudier, J., and R. D. Cole. 1988. Reinvestigation of the sulfhydryl reactivity in bovine brain S-100b (ββ) protein and the microtubule-associated tau proteins. Ca^{2+} stimulates disulfide cross-linking between the S-100β subunit and the microtubule-associated tau(2) protein. *Biochemistry.* 27:2728–2736.

Baudier, J., C. Briving, J. Deinum, K. Haglid, L. Sorskog, and M. Wallin. 1982. Effect of S-100 proteins and calmodulin on Ca^{2+}-induced disassembly of brain microtubule proteins in vitro. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 147:165–167.

Benda, P., J. Lightbody, G. Sato, L. Levine, and W. Sweet. 1968. Differentiated rat glial cell strain in tissue culture. *Science (Wash. DC).* 161:370–371.

Burridge, K. 1986. Substrate adhesions in normal and transformed fibroblasts: organization and regulation of cytoskeletal, membrane and extracellular matrix components at focal contacts. *Cancer Rev.* 4:18–78.

Chung, F.-Z., C.-D. Wang, P. C. Potter, J. C. Venter, and C. M. Fraser. 1988. Site-directed mutagenesis and continuous expression of human β-adrenergic receptors. *J. Biol. Chem.* 263:4052–4055.

Donato, R. 1986. S-100 proteins. *Cell Calcium.* 7:123–145.

Donato, R., and I. Giambanco. 1989. Interaction between S-100 proteins and steady-state and taxol-stabilized microtubules in vitro. *J. Neurochem.* 52:1010–1017.

Donato, R., I. Giambanco, and M. C. Aisa. 1989. Molecular interaction of S-100 proteins with microtubule proteins in vitro. *J. Neurochem.* 53:566–571.

Duncan, A. M. V., J. Higgins, R. J. Dunn, R. Allore, and A. Marks. 1989. Refined sublocalization of the human gene encoding the β subunit of the S100 protein (S100B) and confirmation of a subtle t(9;21) translocation using in situ hybridization. *Cytogenet. Cell Genet.* 50:234–235.

Endo, T., and H. Hidaka. 1983. Effect of S-100 protein on microtubule assembly-disassembly. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 161:235–238.

Giguere, V., S. M. Hollenberg, M. G. Rosenfeld, and R. M. Evans. 1986. Functional domains of the human glucocorticoid receptor. *Cell.* 46:645–652.

Green, P. J., O. Pines, and M. Inouye. 1986. The role of antisense RNA in gene regulation. *Annu. Rev. Biochem.* 55:569–597.

Griffin, W. S. T., L. C. Stanley, C. Ling, L. White, V. MacLeod, L. J. Perrot, C. L. White, and C. Araoz. 1989. Brain interleukin I and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer disease. *Proc. Natl. Acad. Sci. USA.* 86:7611–7615.

Higashida, H., M. Sano, and K. Kato. 1985. Forskolin induction of S-100 protein in glioma and hybrid cells. *J. Cell. Physiol.* 122:39–44.

Hitt, A. L., A. R. Cross, and R. C. Williams. 1990. Microtubule solutions display nematic liquid crystalline structure. *J. Biol. Chem.* 265:1639–1647.

Holley, R. W. 1975. Control of growth of mammalian cells in cell culture. *Nature (Lond.).* 258:487–490.

Holt, J. T., T. Venkat Gopal, A. D. Moulton, and A. W. Nienhuis. 1986. Inducible production of c-fos antisense RNA inhibits 3T3 cell proliferation. *Proc. Natl. Acad. Sci. USA.* 83:4794–4798.

Holt, J. T., R. L. Redner, and A. W. Nienhuis. 1988. An oligomer complementary to c-myc mRNA inhibits proliferation of HL-60 promyelocytic cells and induces differentiation. *Mol. Cell. Biol.* 8:963–973.

Klein, J. R., D. S. Hoon, J. Nagauyan, E. Okun, and A. J. Cochran. 1989. S-100 protein stimulates cellular proliferation. *Cancer Immunol. Immunother.* 29:133–138.

Kligman, D., and D. R. Marshak. 1985. Purification and characterization of a neurite extension factor from bovine brain. *Proc. Natl. Acad. Sci. USA.* 82:7136–7139.

Kligman, D., and L. J. Hsieh. 1987. Neurite extension factor induces rapid morphological differentiation of mouse neuroblastoma cells in defined medium. *Dev. Brain Res.* 33:296–300.

Kuwano, R., H. Usui, T. Maeda, T. Fukui, N. Yamanari, E. Ohtsuka, M. Ikehara, and Y. Takahashi. 1984. Molecular cloning and the complete nucleotide sequence of cDNA to mRNA for S100 protein of rat brain. *Nucleic Acids Res.* 12:7455–7465.

Labourdette, G., and P. Mandel. 1980. Effect of norepinephrine and dibutyryl cAMP on S-100 protein level in C6 glioma cells. *Biochem. Biophys. Res. Commun.* 96:1702–1709.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265–275.

Marks, A., D. Petsche, D. O'Hanlon, P. C. Kwong, R. Stead, R. Dunn, R. Baumal, and S. K. Liao. 1990. S100 protein expression in human melanoma cells. Comparison of levels of expression among different cell lines and individual cells in different phases of the cell cycle. *Exp. Cell Res.* 187:59–63.

Mellon, P., V. Parker, Y. Gluzman, and T. Maniatis. 1981. Identification of DNA sequences required for transcription of the human α1-globin gene in a new SV40 host-vector system. *Cell.* 27:279–288.

Schnapp, B. J. 1986. Viewing single microtubules by video light microscopy. *Methods Enzymol.* 134:561–573.

Shashoua, V. E., G. W. Hesse, and B. W. Moore. 1984. Proteins of the brain extracellular fluid: evidence for release of S100 protein. *J. Neurochem.* 42:1536–1541.

Suzuki, F., K. Kato, T. Kato, and N. Ogasawara. 1987. S-100 protein in clonal astroglioma cells is released by adrenocorticotropic hormone and corticotropin-like intermediate-lobe peptide. *J. Neurochem.* 49:1557–1563.

Tsunamoto, K., S. Todo, S. Imashuku, and K. Kato. 1988. Induction of S100 protein by 5-bromo-2′-deoxyuridine in human neuroblastoma cell lines. *Cancer Res.* 48:170–174.

Van Eldik, L. J., and D. B. Zimmer. 1987. Secretion of S-100 from rat C6 glioma cells. *Brain Res.* 436:367–370.

Van Eldik, L. J., and D. B. Zimmer. 1988. Mechanisms of action of the S100 family of calcium modulated proteins. *In* Calcium and Calcium Binding Proteins. C. H. Gerday, R. Gilles, and L. Bolis, editors. Springer-Verlag, Berlin. 114–127.

Van Eldik, L. J., J. L. Staecker, and F. Winningham-Major. 1988. Synthesis and expression of a gene coding for the calcium-modulated protein S100β and designed for cassette-based, site-directed mutagenesis. *J. Biol. Chem.* 263:7830–7837.

Welsh, M. J. 1983. Localization of calmodulin and calmodulin acceptor sites by fluorescence methods. *Methods Enzymol.* 102:110–121.

Wigler, M., A. Pellicer, S. Silverstein, and R. Axel. 1978. Biochemical transfer of single-copy eukaryotic genes using total cellular DNA as donor. *Cell.* 14:725–731.

Winningham-Major, F., J. L. Staecker, S. W. Barger, S. Coats, and L. J. Van Eldik. 1989. Neurite extension and neuronal survival activities of recombinant S100β proteins that differ in the content and position of cysteine residues. *J. Cell Biol.* 109:3063–3071.

Zimmer, D. B., and L. J. Van Eldik. 1988. Levels and distribution of the calcium-modulated proteins S100 and calmodulin in rat C6 glioma cells. *J. Neurochem.* 50:572–579.

Zimmer, D. B., and L. J. Van Eldik. 1989. Analysis of the calcium-modulated proteins, S100 and calmodulin, and their target proteins during C6 glioma cell differentiation. *J. Cell Biol.* 108:141–151.