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EXPERIMENTAL STUDIES

Stable Transfection of DAOY Cells with a GM3 Synthase Antisense Construct and Transient Reduction in Ganglioside Content

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Abstract—Tumor cell gangliosides are bioactive molecules involved in tumor-host interactions. To investigate their role in tumor formation and angiogenesis, we sought to develop an inhibitory model targeting human GM3 synthase, an essential enzyme in the ganglioside synthesis pathway, by antisense transfection. We prepared a number of transfectants from DAOY human medulloblastoma cells and isolated clones that stably expressed a 560-bp fragment of human GM3 synthase cDNA, in either sense or antisense orientation, as well as clones transfected with an empty vector. Both sense and antisense clones permanently incorporated mammalian expression vectors into their genomes. The DAOY cell clones were screened for ganglioside content using total lipid extraction, ganglioside isolation, and HPTLC. One antisense-transfected clone, 7.2A, in which total ganglioside content was reduced by 70%, was selected for further study. All sense- and sham-transfectants had ganglioside levels not different from that of untransfected DAOY cells. After 10 passages however, while antisense mRNA expression was fully maintained, the ganglioside content of 7.2A cells had reverted to normal levels. Antisense RNA transfection can sometimes have a reversible effect on the expression of a target. Possible regulatory mechanisms of this previously unrecognized process of reversion to wild type phenotype are discussed.

Key words: gangliosides, GM3 synthase, antisense mRNA, transfection, medulloblastoma.

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INTRODUCTION

Gangliosides are glycosphingolipids containing sialic acid residues. They reside in mammalian cell membranes, and are particularly enriched in neuronal cell membranes [1]. During the past decades, the shedding of gangliosides into circulation by tumors became a well-recognized phenomenon [2, 3]. Gangliosides have a number of functional properties, including immunosuppressive, tumor-enhancing, angiogenic, and signaling (via modulation of growth factor responses) [4].

Here we report that we succeeded in obtaining a stable antisense-transfected DAOY clone with substantially reduced total ganglioside content. However, the reduction was temporary and the clone reverted to a normal level of expression of gangliosides despite permanent expression of GM3 synthase antisense mRNA. To the best of our knowledge, such reversion of an antisense effect has not been reported previously. This finding has several implications, namely, that stable antisense RNA transfection does not always ensure a similarly stable inhibitory effect, and that the phenotype of any successful antisense clones should be carefully monitored in subsequent experiments, especially as the number of passages in culture increases.

METHODS

Preparation of stable transfectant cell lines. The total RNA was extracted from DAOY (human medulloblastoma) cells using the RNeasy mini kit (Qiagen), and first strand cDNA was generated using an RT-PCR kit (Stratagene). The cDNA sequence for GM3 synthase was obtained from GenBank (accession number AB018356). PCR primers were designed based on that sequence using Primer 3 web-based software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and synthesized by Integrated

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Abbreviations used: Cpm—counts per minute, HPTLC—high performance thin-layer chromatography, LBSA—lipid bound sialic acid, RT-PCR—reverse transcription and PCR, PBS—phosphate buffered saline.

DNA Technologies, Inc. Each primer has a restriction enzyme site added to the 5' end that is also found at the multiple cloning site of the vector to allow insertion in a known orientation (sense or antisense). The ~530 bp region of GM3 synthase cDNA, 188–721, covering translation start (position 278) was amplified using PCR.

Universal left primer carries an Mlu I restriction site at its 5' end. The primer designed for the antisense construct carries an Nhe I restriction site at its 5' end and the one designed for the control sense construct carries a Sal I restriction site. This ensured opposite orientation of the antisense and control sense inserts in pCI-neo vector. PCR products were gel-purified and extracted from agarose using the Qiaex II kit (Qiagen) and then digested using Mlu I and either Nhe I (antisense) or Sal I (sense).

Antisense Vector Assembly. The 5.5-kb pCI-neo mammalian expression vector (Promega) was digested with Mlu I and either Sal I or Nhe I, and gel-purified sense or antisense inserts were ligated into the vector. Ligation mixtures were used to transform XLI-Blue competent cells, and recombinant clones were identified using Plasmid Minipreps (Qiagen) and restriction analysis. Inserts and correct orientation were confirmed by sequencing at the University of Chicago Cancer Research Center Sequencing Facility.

Transfection and selection of stable transfectants. DAOY cells (human medulloblastoma) were transfected with the antisense vectors using electroporation (Gene Pulser II, BioRad, CA) and seeded in 10 cm-diameter culture dishes containing complete EMEM, supplemented with 10% FBS. In addition, sense controls were prepared in a similar manner and, as a sham control, one aliquot of DAOY cells was transfected with the empty pCI-neo vector (without inserts), and another with saline instead of DNA solution. After 48 h, 400 ng/ml of the selection drug, antibiotic geneticin (G418), was added to each plate and the colonies were selected after on day 11. Colonies were initially placed into 6-well plates until confluent and then transferred to 25 cm^2 cell culture flasks, followed by subculturing in 75 and 175 cm^2 flasks. Since the antisense construct and geneticin resistance gene (Neo) are linked in the integrated pCI-neo vector, the above selection method allowed for isolation of the cells that: (a) have permanently incorporated the pCI-neo vector into their genome (multiple copies) and (b) express sufficient levels of the drug-resistance enzyme and antisense mRNA. Upstream of neomycin phosphotransferase gene is the SV40 enhancer and early promoter, and the CMV immediate-early enhancer/promoter region associated with the multiple cloning site allows for strong, constitutive expression in a number of cell types. The control cells transfected with saline did not survive drug selection. Aliquots of each transfectant cell clone were frozen down for further stepwise analysis.

Ganglioside isolation and quantitation. Seventeen drug-resistant cell clones (twelve cDNA-based antisense clones and three sense controls, as well as non-transfected DAOY cells, and sham control clones) were grown to approximately 20×10^6 cells. Confluent DAOY cells were harvested by trypsinization and aliquots were taken for total cell protein assay (BioRad, CA). The screening strategy was as follows: gangliosides in the antisense (and sense) transfectants and controls were isolated by chloroform/methanol extraction and purified as described previously [5]. The ganglioside composition was analyzed by high-performance thin-layer chromatography and gangliosides were visualized with resorcinol reagent [5]. Densitometric analysis of the TLC plates was performed using Scion Image software (NIH, Bethesda, MD) and total ganglioside content of each transfectant clone calculated per 10^8 cells. As a second quantification, total ganglioside content of the clones was also calculated per microgram of total cell protein.

Protein assay. Samples consisting of 2.5×10^4 cells in PBS were lysed with 1/3 volume 1 M NaOH and incubated for 5 days (vortexed once per day). Then, the protein assay was performed according to instructions for BioRad DC Protein Assay (BioRad, CA).

RT-PCR and genomic DNA assay. Clones SH1, 3.2S, 7.2A, 6.2A (the latter is an antisense-transfected clone with normal ganglioside content), and untransfected DAOY cells were grown to confluence in 75 cm^2 flasks, and extraction of RNA and DNA were performed according to a protocol provided with TRIzol reagent (Invitrogen, CA). About 1 μg of RNA from each clone was used to prepare first-strand cDNA using ImProm-II Reverse Transcriptase (Promega, WI). The resulting cDNA and genomic DNA were analyzed using regular PCR for the presence of the antisense or sense cassette. 2.5 μl of the unpurified cDNA reaction or genomic DNA solution were then combined with 12.5 μl PCR master mix (Promega, WI), 9 μl PCR-grade water, and 0.5 μl of each primer. Primers specific for pCI-neo vector with a sense or antisense insert were designed using Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and synthesized by IDT DNA, IN. Right primer: GCTCGAAGCATTAACCCTCACTAAAGG; left antisense primer: CCTCCGCTTCCAATAACCACACAG; left sense primer: AGCAATGCCAAGTGAGTACACCTATGTG. The PCR products were analyzed using 1% agarose gel electrophoresis with ethidium bromide staining.

Metabolic radiolabeling of cellular gangliosides. GM3 synthase antisense and sense transfected DAOY cells (clones 7.2A and 3.2S) were cultured in EMEM medium supplemented with 10% FBS and 0.1 mg/ml geneticin in 75 cm^2 flasks until subconfluent. Then 1.6 μCi of D-[6-^{3}H]glucosamine hydrochloride (30.9 Ci/mmol) and D-[6-^{3}H]galactose (35 Ci/mmol, New England Nuclear, MA) were added per milliliter of culture

medium, the cells were cultured for 48 h, and washed with fresh medium. One set of flasks was harvested, while the other was left to grow for another 48 h in the regular medium without isotopes and then harvested too. After that, cell pellets were processed for ganglioside purification as described above. The radiolabeled gangliosides were quantified using a Beckman LS-6500 multipurpose scintillation counter.

Quantitation of antisense mRNA. Cells of clone 7.2A at passages 9 and 13 as well as one of the antisense clones (6.2A) with an almost normal ganglioside level, were grown to confluence in T-25 cell culture flasks and RNA was isolated using Trizol reagent (Invitrogen, CA). 2 μg of RNA from each of the three samples were analyzed semi-quantitatively for the level of GM3 synthase antisense mRNA expression using IntraSpec Comparative RT-PCR kit (Ambion, TX). In brief, [α-^{32}P]dATP labeled first strand cDNA was synthesized using primers Tag10RT, Tag50RT, and Tag10RT (supplied with the kit) for clones 6.2A, 7.2A passage 9, and 7.2A passage 13, respectively. Primers Taq10RT and Tag50RT add tails of different lengths (the former is 40 bp shorter than the latter) to 3' ends of cDNA, in such a way that the all cDNAs in clone 7.2A passage 9 are 40 bp longer than those in clone 6.2A and 7.2A passage 13. Since pairwise mixtures of cDNAs will compete for the same primers in each PCR, different intensity of the bands in an agarose gel should reveal differences in the amounts of GM3 synthase antisense mRNA in the three samples. Each first strand reaction was purified using NucAway columns (supplied with the kit) and cDNA present in eluates was quantified by scintillation counting (Beckman LS-6500). Equal amounts (based on cpm) of each cDNA were mixed pairwise and amplified by PCR using Outer Tag primer (supplied with the kit) and primer AmbAS: ACTATAGGCTAGCATGTCCA (specific for the antisense construct in that it covers part of the expression vector in the promoter area and part of the GM3 synthase antisense insert). We used PCR Master Mix (Promega, WI) for the amplification and used the following conditions: per 25 μl PCR reaction we mixed 12.5 μl PCR Master Mix, 3 μl of 2 μM Outer Taq primer, 0.5 μl of 50 μM AmbAS primer, 3 μl water and about 6 μl of each equimolar pairwise mixture of cDNA eluates from the antisense clones. The first mixture contained 2.4 and 3.5 μl of eluates from clones 6.2A and 7.2A passage 9 respectively. The second mixture contained 3.5 and 2.9 μl of eluates from clones 7.2A passage 9 and 7.2A passage 13 respectively. The third mixture contained 2.4 and 2.9 μl of eluates from clones 6.2A and 7.2A passage 13 respectively. Cycling conditions were (35 cycles total): first denaturation at 95°C for 2 min; all subsequent denaturation steps at 95°C for 30 s; annealing at 50°C for 30 s; extension at 72°C for 45 s; final step hold at 4°C. All PCRs were run on an MJ Research Gradient Cycler PTC-225 with a heated lid. Control PCR reactions containing one cDNA only from each clone were also run in a similar manner. The products were analyzed by electrophoresis in 2% agarose gels containing ethidium bromide.

RESULTS AND DISCUSSION

Ganglioside content of DAOY cell clones. Seventeen drug-resistant clones of DAOY cells transfected with antisense (12 clones), sense (3 clones), or empty plasmids (2 clones) were analyzed for total ganglioside content. Shown in Fig. 1 (upper panel) are the HPTLC ganglioside patterns of seven clones, and Fig. 2 summarizes total ganglioside counts of all 17 clones studied and two measurements of untransfected DAOY cells. One of twelve antisense clones, 7.2A, had an approximately 70% reduction in the ganglioside concentration at passage 5, compared to sense and sham controls (Fig. 2). This clone, 7.2A, did not show any obvious qualitative change in the ganglioside complement (Fig. 1, upper panel). The degree of inhibition of ganglioside synthesis was not as pronounced in other antisense clones (Figs. 1, upper panel and Fig. 2).

The antisense clone 7.2A was analyzed further at a later passage (eight) and the inhibitory effect of GM3 synthase antisense transfection on cellular gangliosides, namely, the ~ 70% reduction of ganglioside level, was confirmed (Fig. 1, lower panel). This was established both on a per cell basis (46 nmol LBSA per 10^8 cells) and as content per unit of total cell protein (0.85 nmol LBSA per mg cell protein), compared to control DAOY cells, (148 nmol LBSA per 10^8 cells (average of two measurements, Fig. 2) or 2.7 nmol LBSA per mg of cell protein). These data are consistent with the concentrations reported previously for DAOY cells [6, 7].

Antisense RNA expression. To confirm that the inhibitory effect on ganglioside content was caused by GM3 antisense transfection, we analyzed the expression of antisense mRNA in clone 7.2A (Fig. 3, upper panel). RT-PCR assay demonstrated that at passage 9, antisense mRNA was expressed in the antisense clone 7.2A (Fig. 3, upper panel), while the sense mRNA fragment was expressed in a randomly chosen sense transfected clone. Both the antisense and sense mRNA species were absent from sham-transfected (empty plasmid), untransfected DAOY cells (Fig. 3, upper panel). Proliferation assay showed no difference in the rate of cell division between antisense clone 7.2A and sense clone 3.2S at passage 9 and was consistent with previously published data for DAOY cells, doubling time of about 24 h [6].

Assessment of cellular ganglioside synthesis. For subsequent characterization of the antisense inhibition, we investigated the GM3 synthase enzyme activity in the antisense and sense clones using a metabolic radiolabeling assay (Fig. 4). These studies were conducted on cells at passage 13. Surprisingly, the assay did not show a significant difference in enzyme activity between the antisense clone 7.2A and a control sense

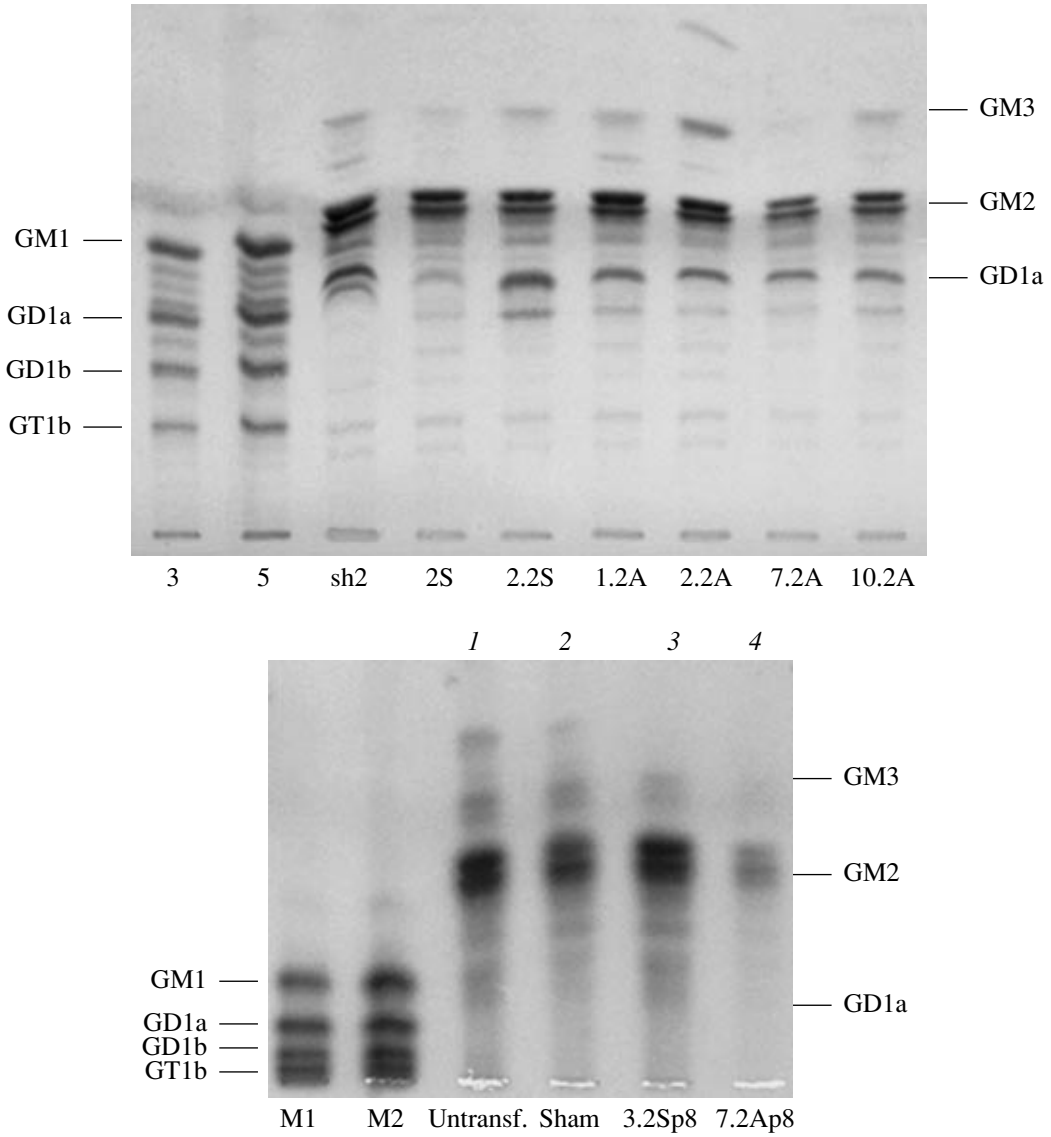

Fig. 1. HPTLC analysis of gangliosides, isolated from passages 5 and 8 of antisense and sense transfected DAOY cells. Gangliosides from 3×10^6 cells were spotted in each lane. Upper panel, passage 5: 3 and 5 correspond to 3 and 5 nmol LBSA of human brain gangliosides (markers); sh2—sham control; 2s, 2.2S-sense transfected DAOY cells; 1.2A, 2.2A, 7.2A, 10.2A-antisense transfected clones. Bottom panel, passage 8: M1 and M2 correspond to 3 and 6 nmol LBSA of bovine brain gangliosides (markers); Lane *1*—untransfected DAOY cells; Lane *2*—sham control cells; Lane *3*—sense transfected clone 3.2S; Lane *4*—antisense transfected clone 7.2A (passage 8).

clone (Fig. 4, left panel), suggesting that the antisense effect had disappeared by this time. This prompted us to analyze ganglioside content and antisense RNA expression of clone 7.2A again.

Further analysis of clone 7.2A. Because of the surprisingly unchanged rate of ganglioside synthesis noted above, we analyzed ganglioside content of clone 7.2A for the third time, this time at passages 10 and 13 (passage 10 is shown in Fig. 4, right panel). This analysis revealed that the antisense clone 7.2A had reverted to the wild type level of gangliosides beginning at passage 10. This sudden disappearance of the antisense effect (between passages 8 and 10, see Fig. 1, bottom panel and Fig. 4, right panel) did not affect drug-resistance of clone 7.2A, since all clones except untransfected DAOY cells were maintained in a medium containing 0.1 mg/ml geneticin (G418). In addition, PCR analysis (Fig. 3, bottom panel) revealed both that the antisense cassette was present in the genome of 7.2A cells, and that antisense mRNA was still expressed at passage 13.

Quantitation of antisense mRNA expression. To establish whether the disappearance of antisense effect was due to a change in antisense mRNA expression in different passages of clone 7.2A, we used semi-quantitative (comparative) RT-PCR to compare two passages of clone 7.2A and an antisense-transfected clone 6.2A

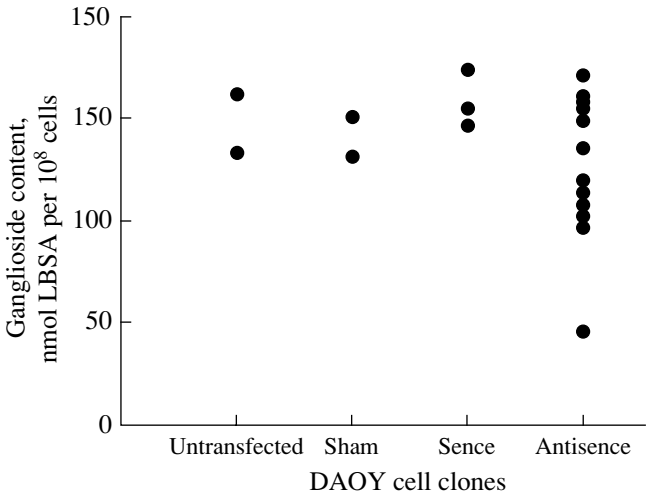

Fig. 2. Densitometric quantitation of HPTLC data. Ganglioside content of various DAOY clones transfected with GM3 synthase antisense, sense or empty vector is shown as a dot for each cell clone.

that does not have a reduction in cellular gangliosides. The earliest passage of 7.2A that was available was passage 8 (also the last passage that was confirmed to have phenotypic reduction in gangliosides), the earliest passage available for mRNA quantitation was passage 9 (passage 8 grown to a confluent T-25 flask and harvested). We performed pairwise comparisons of antisense mRNA concentrations between cDNAs isolated from these three cell populations: clone 7.2A passage 9, clone 7.2A passage 13, and clone 6.2A passage 8. Figure 5 shows that pairwise comparison in these clones revealed roughly similar antisense mRNA levels in passages 9 and 13 of clone 7.2A. Clone 6.2A had almost the same level of antisense mRNA as that of clone 7.2A (Fig. 5, lane 1), and antisense mRNA expression did not decrease from passage 9 to 13 of clone 7.2A (Fig. 5, lane 2).

These results demonstrate that the antisense clone (7.2A), which had a ~70% reduction in total cell gangliosides at passages 5 and 8, lost the antisense effect by passage 10 despite the continued neomycin-resistance and expression of antisense mRNA.

A ganglioside knockdown model is necessary for functional studies of tumor cell gangliosides and could be used for elucidating the effects of gangliosides on tumor formation and angiogenesis in vivo and in vitro. This paper presents the first successful knockdown of human GM3 synthase, an entry-level enzyme in the pathway of human ganglioside biosynthesis. While GM3 synthase and a number of other enzymes in ganglioside biosynthetic pathway, such as GD3 synthase and glucosylceramide synthase, have been successfully knocked down or knocked out in mice [8], a successful downregulation of human GM3 synthase has not been reported previously, and no chemical inhibitor or specific genetic model exists for human GM3 synthase at the time of this writing. On the other hand, successful inhibition of human GM3 synthase could potentially provide researchers with a tool to study the function of tumor cell gangliosides.

As shown above, one of the antisense clones that we obtained, 7.2A, had approximately 70% reduction in total ganglioside level as confirmed by two lipid analyses, at passages 5 and 8. This inhibitory effect on GM3 synthase activity was only temporary however, and, despite stable expression of antisense RNA, clone 7.2A reverted to a normal level of cell gangliosides after passage 10 in culture. A finding that the phenotypic effect of stable transfection with antisense RNA was reversed has not been reported in literature to the best of our knowledge. It has been demonstrated previously [9] that stable antisense transfection often produces a number of clones expressing antisense RNA but showing no phenotypic effect, as was also the case in this work. However, the reversion of a successful stable antisense clone to wild type phenotype has not been reported to date. In fact, in the specific case of glucosylceramide synthase antisense transfectants, antisense transfected clones retained inhibitory effect on gangliosides for several years and many passages in culture [10].

Attempts to clarify possible mechanisms of such a reversion warrant a brief excursion into the history and the current understanding of mechanisms of action of antisense RNA. First reports of successful downregulation of gene expression using endogenously expressed or synthetic RNA appeared around 1985 [11]. This coincided with the discovery of the regulatory role of natural antisense RNAs in mobile elements and bacteria [12]. The main mechanism of action of these natural RNAs in bacteria was believed to be a binding to the target mRNA and preventing translation. Since then, a number of naturally occurring antisense RNAs to protein-coding genes in higher organisms including human have been described [13]. The function of these RNAs, however, with respect to regulation of sense mRNA expression and processing, has yet to be identified. Among several potential mechanisms are the action of double-stranded RNA-specific adenosine deaminase; double-strand RNA binding and stabilizing protein factors; double-strand RNases [14]; and competitive inhibition at various stages of gene expression, such as RNA transcription, processing, transport, and translation [15]. Double-stranded RNA adenosine deaminase is believed to be a multi-enzyme complex that recognizes RNA duplexes and deaminates adenosines within such regions [14]. This results in a destabilization of RNA:RNA interactions such as those involved in splicing, RNA editing and translation, and ultimately leads to disruption of the expression of a gene in question [14]. In conjunction with this finding, several protein factors were described that can protect RNA duplexes from modification by double-stranded RNA adenosine deaminase, and thus can also be involved in the antisense mechanisms of gene expression regulation [16].

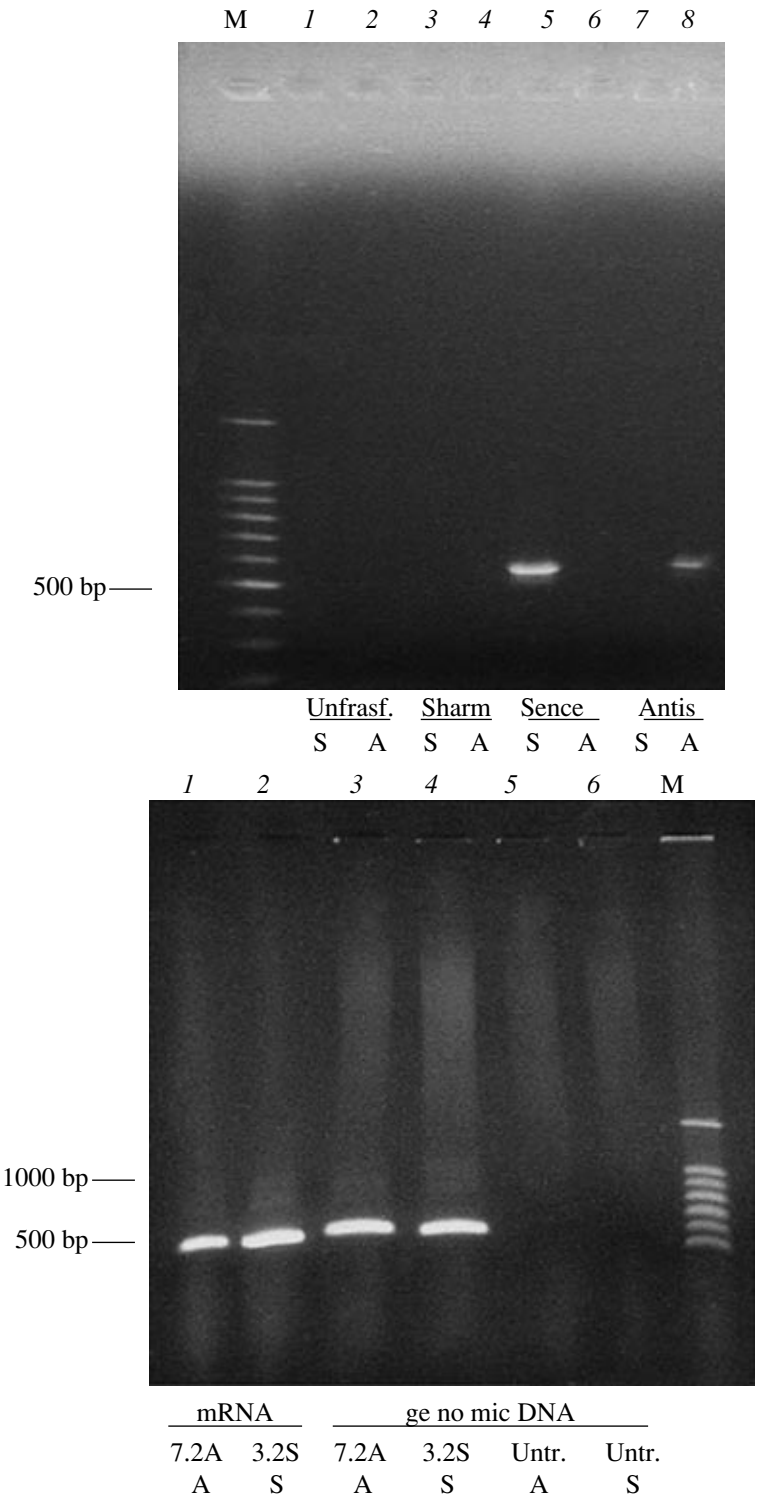

Fig. 3. RT-PCR analysis of antisense mRNA expression in passages 9 and 13 of clone 7.2A and controls.
In addition, PCR analysis of genomic DNA from passage 9 of clone 7.2A for presence of GM3 synthase antisense cassette with appropriate controls is shown as well. For all reactions, a common right primer was used. A left primer specific for the sense cassette or transcript is shown as S in figure, and a left primer specific for antisense cassette or transcript is shown as A. Only one or the other was used in each reaction. Upper panel, passage 9: M—DNA molecular weight markers (Promega); *1*—mRNA from untransfected cells, primer S; *2*—the same with primer A; *3*—mRNA from sham-transfected cells, primer S; *4*—the same with primer A; *5*—mRNA from sense clone 3.2S, primer S; *6*—the same with primer A; *7*—mRNA from antisense clone 7.2A (passage 9), primer S; *8*—the same with primer A. Bottom panel, passage 13: *1*—mRNA from antisense clone 7.2A (passage 13), primer A; *2*—mRNA from sense clone 3.2S (passage 13), primer S; *3*—genomic DNA from antisense clone 7.2A (passage 13), primer A; *4*—genomic DNA from sense clone 3.2S (passage 13), primer S; *5*—genomic DNA from untransfected DAOY cells, primer A; *6*—genomic DNA from untransfected DAOY cells, primer S; M—DNA molecular weight markers (Promega, WI).

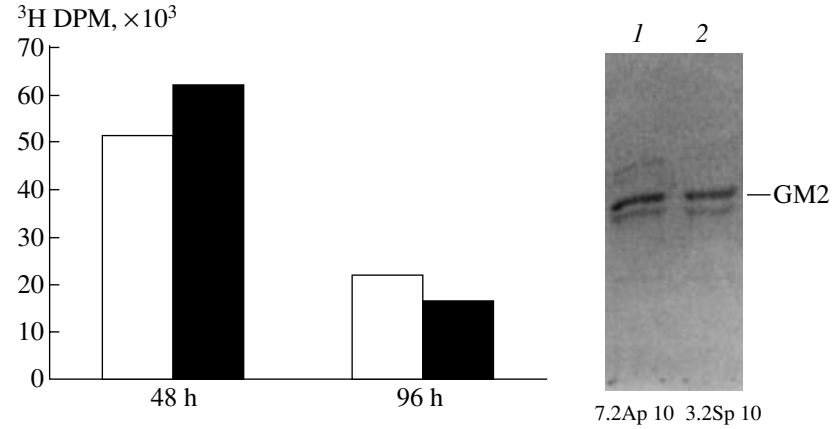

Fig. 4. Metabolic radiolabeling of cellular gangliosides (GM3 synthase activity assay) in antisense and sense transfected DAOY cells (passage 13). Left panel: Each bar represents 1.8×10^6 cells; light bars—antisense clone 7.2A, dark bars—sense clone 3.2S. Passage 13 cells were cultured in the presence of 1.6 μCi/ml of D-[6-^{3}H]glucosamine hydrochloride (30.9 Ci/mmol) and D-[6-^{3}H]galactose for 48 h, then part of them was harvested and ganglioside-associated activity analyzed (bars shown at 48 h). The remaining cells were washed free of the label and cultured for an additional 48 h, then harvested and analyzed in the same manner (bars shown at 96 h). The right panel shows the results of ganglioside quantitation in clone 7.2A passage 10. Lane *1*—antisense transfected clone 7.2A (passage 10); Lane *2*—sense transfected clone 3.2S.

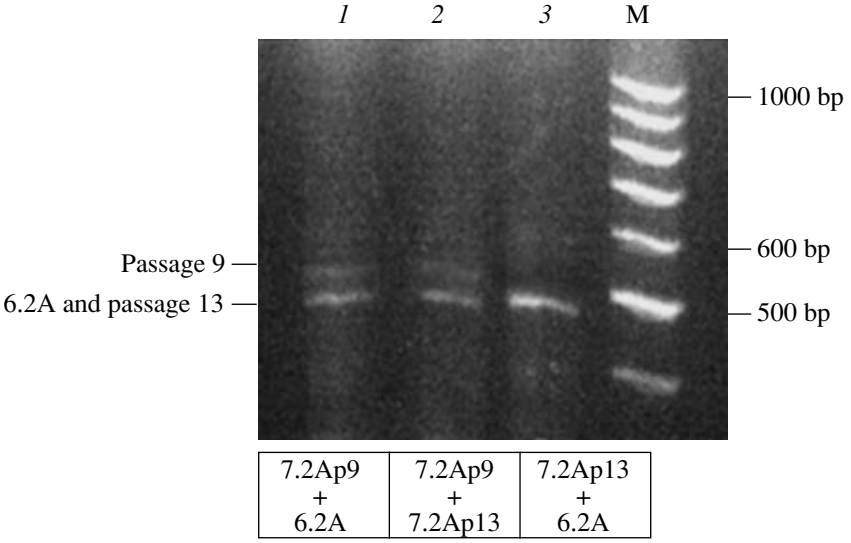

Fig. 5. Comparative RT-PCR assay of antisense mRNA expression in different antisense clones and passages. Equal amounts of cDNA from clones 6.2A passage 8, 7.2A passage 9, and 7.2A passage 13 were mixed pairwise and amplified with primers specific for GM3 synthase antisense construct. cDNAs were prepared using IntraSpec Comparative RT-PCR Kit (Ambion, TX) in such a way that the all cDNAs in clone 7.2A passage 9 are 40 bp longer than those in clone 6.2A and 7.2A passage 13. Since pairwise mixtures of cDNAs will compete for the same primers in each PCR, different intensity of the bands will reveal differences in the amounts of GM3 synthase antisense mRNA in the three samples. Lane *1*—antisense mRNA from clone 7.2A passage 9 (upper band) compared to that of clone 6.2A passage 8 (lower band). Lane *2*—antisense mRNA from clone 7.2A passage 9 (upper band) compared to that of clone 7.2A passage 13 (lower band). Lane *3*—mixture of clones 7.2A passage 13 and 6.2A passage 8 (one band). M: markers of DNA molecular weight (Promega, WI).

Until a recently, little was known about double-stranded RNases in mammalian cells; in bacteria, one such well-characterized enzyme is RNase III [17]. With the explosion of data on RNA interference (RNAi) in mammalian cells [18], double-stranded RNase activity, such as RNase III-like human dicer enzyme [19], was identified in the nucleus and cytoplasm of human cells. The mechanism of gene silencing through RNAi was studied in *Drosophila* cells in vitro, and appears to be mediated by a large multi-enzyme complex that is activated by short double-stranded RNA duplexes (21–25 bp) and is recruited to the mRNA of the matching

sequence, which results in its cleavage and degradation [20]. There are no data suggesting that antisense RNA expressed endogenously can trigger the RNAi mechanism. However, it can be hypothesized that formation of extended duplex regions involving the antisense and target RNA could touch off cleavage by dicer, resulting in small interfering RNAs and the recruitment of RNA-induced silencing complex (RISC).

Much of the efforts of targeting various stages of RNA processing through non-cleaving mechanisms involved synthetic deoxy- and ribooligonucleotides as opposed to introduction of transgenes expressing antisense mRNA [15]. These experiments with antisense oligonucleotides revealed that antisense downregulation of a target mRNA level can involve inhibition of capping, splicing, polyadenylation, export from the nucleus (only indirectly via the previous three mechanisms), and various stages of translation [15]. Relevance of the described effects to the mechanisms involved in the antisense effect of endogenously expressed RNAs of substantial length (100 to 800 nucleotides) has yet to be established.

Despite widespread use, the actual mechanism involved in antisense inhibition following transfection of cells with antisense transgenes encoding mRNA, which is complementary to portions of sense mRNA, remains unclear [9]. What little that is known suggests that in cases of successful application of this approach, up to 95% of the sense mRNA can be bound to the antisense RNA in the nucleus, and the total amount of the target mRNA is significantly reduced [21]. Some data suggest that for successful downregulation of the target gene expression, a 10 to 50-fold excess of antisense RNA over sense mRNA may be required [22]. Total length antisense cDNA was shown to be ineffective [23], and generally a shorter (100–500 bp) region within the target mature mRNA has to be found (using the trial-and-error approach) that would lead to successful antisense downregulation of the gene of interest [9]. Some investigators have previously reported successful downregulation of genes using antisense constructs targeting splice junctions of a primary transcript, possibly via prevention of the nuclear export of the unprocessed RNA [15]. In this work, we identified a clone with a substantial reduction of cell ganglioside content among twelve cDNA-based antisense transfectants, where we targeted a region of about 530 bp of GM3 synthase cDNA (spliced mRNA).

In many reports [24, 25], only a minority of antisense transfected clones show signs of inhibition of a target gene, despite active expression of antisense mRNA. This was apparently the case in our study as well. A possible explanation for such heterogeneity could be the presence of different copy numbers of the antisense transgenes in the genomes of transfected cells, which would enable certain clones to achieve the high level of antisense mRNA expression necessary for an antisense mechanism to take effect. However, this does not explain the reversion of the inhibitory effect in a later passage of a clone, and our analysis revealed that the reversion of the phenotypic antisense effect was not accompanied by a decrease in antisense mRNA expression. The rapidity of the antisense reversion (within 2 passages, from passage 8 to 10) excludes the possibility that a mutation in the antisense gene cassette could have caused this reversal, because it would have had to happen in millions of cells almost simultaneously.

We can hypothesize that the antisense RNA mechanism is dynamically regulated at the epigenetic level [25] and may involve up- and downregulation of certain double-stranded RNA modifying enzymes and binding factors, caused by the presence of antisense RNA or duplex RNA, or their degradation products [14]. In addition, subtle differences in the levels of expression or functionality of these factors between different clones of highly mutable tumor cells, such as DAOY human medulloblastoma cells, could lead to different responsiveness of these clones to antisense mRNA.

Permanent transfection of tumor cells with antisense RNA may not always result in a stable inhibitory effect, and therefore the phenotype of successful antisense clones should be periodically reconfirmed, particularly, before and after subsequent experiments that test the functional impact of the transfection.

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