Headline Articles

Enhancement of the Antisense Effect of Polysaccharide–Polynucleotide Complexes by Preventing the Antisense Oligonucleotide from Binding to Proteins in the Culture Medium[#]

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Schizophyllan is a β-(1→3)-D-glucan existing as a triple helix in water and as a single chain in dimethyl sulfoxide (DMSO), respectively. As we already reported, when a homo-phosphodiester-polynucleotide is added to the schizophyllan/DMSO solution and, subsequently, DMSO is exchanged for water, the single chain of schizophyllan (s-SPG) forms a complex with the polynucleotide. In this paper, we report that phosphorothioate oligonucleotides can form a complex with s-SPG in the same manner as phosphodiester oligonucleotides. We carried out an in vitro antisense assay combining melanoma cell lines and a phosphorothioate antisense oligonucleotide (AS ODN) to depress *c-myb* mRNA. We found that the AS ODN bound to the complex reduces cell growth more efficiently than that of naked AS ODN by preventing the AS ODN from binding to albumin in the culture medium and being hydrolyzed.

Antisense oligonucleotides (AS ODNs) have been studied as a noteworthy strategy to suppress a particular gene expression.[1–3] Principles of antisense therapy are (1) to deliver AS ODN to the target cell, (2) to induce endocytosis of AS ODN, (3) to bind AS ODN to a particular mRNA to make a DNA/RNA duplex, and (4) to make RNase H cleave the duplex so as to inhibit protein expression. The major advantage of this strategy over conventional drugs is in the potential specificity of the action. Theoretically, an oligonucleotide can be designed to target any single gene in the human genome. Therefore, inhibition at the genetic level should be a more efficient intervention in the disease process than inhibition at the protein level. However, there are two major issues to overcome. The instability of AS ODNs in serum and the low up-take efficiency into the target cells.[3] The instability of AS ODNs is mainly ascribed to two factors: the hydrolysis mediated by deoxyribonuclease and non-specific binding to the serum proteins. The hydrolysis can be overcome by use of oligonucleotide analogues such as phosphorothioates, phosphoramidates, and peptide nucleic acids.[4] In particular, phosphorothioates are the leading candidate among

the first generation of antisense compounds. Several of them are currently in phase I/II clinical trials.[5,6] Fortunately, phosphorothioates have an unexpected ability regarding cellular up-take, and currently, it is believed that cells acquire them though a receptor mediated endocytosis.[4,7]

Non-specific binding to proteins is the major obstruction in the case of delivering phosphorothioate AS ODN to the target cell. Several materials that can form a complex with phosphorothioates have been studied as AS ODN carriers.[3] Cationic lipids can form a complex with AS ODNs and encapsulate them in the liposome.[3,8] The liposomal delivery is one of the preferred methods, however, there are some drawbacks in its use. For instance, cationic liposomes tend to accumulate in the reticuloendothelial system, leading to a short life time in the serum. Furthermore, the size of the liposome is usually too large for cells.[3,8] Synthetic polycations, such as poly(L-lysine) and polyethyleneimine (PEI) have been studied as an AS ODN carrier, because polycations can form polyion complexes with polynucleotides.[3] Although polycations have a great advantage in improving transfection,[3,8] serious drawbacks have been pointed
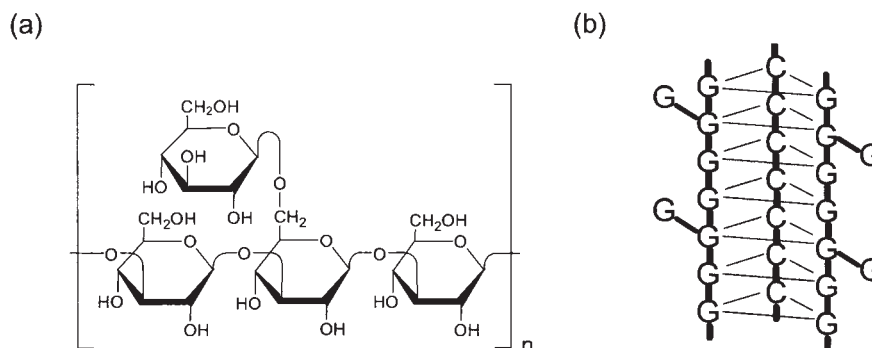
Fig. 1.   Repeating units of schizophyllan (a), and the proposed interaction scheme based on our stoichiometry (b).[16] G and C denote the glucose and cytosine in the chains, respectively.

out, such as toxicity of the polycations and poor solubility of the resultant polyion complexes.[3,8] Graft-copolymers or block-copolymers consisting of polyethylene glycol and polycations have been proposed to improve the solubility.[9–12] However, cytotoxicity still remains a serious problem. Utilizing natural polysaccharides is considered to be a good and new approach to design an AS ODN carrier.[12] This is because natural polysaccharides can biodegrade into nontoxic components, provide solubility,[13] and be used as a cell-specific ligand.[14] Polysaccharides themselves can be used as an AS ODN binding moiety, because some glucans such as schizophyllan can form a complex with polynucleotides.[15,16]

Schizophyllan is an extracellular polysaccharide produced by the fungus *Schizophyllan commune*. The main chain consists of β-(1→3)-D-glucan and one β-(1→6)-D-glycosyl side chain links to the main chain every three glucose residues (see Fig. 1(a) for the chemical structure).[17] Schizophyllan adopts a triple helical conformation in water and a random coil in dimethyl sulfoxide (DMSO).[18–20] When water is added to the DMSO solution (renaturation), the triple helical structure can be partially retrieved, although the entire chain structure is not the same as that of the original triple helix.[21]

Recently, we found that the single chain of schizophyllan (s-SPG) forms a macromolecular complex with some homo-phosphodiester-polynucleotide [such as poly(C), poly(A), poly(U), poly(dA), and poly(dT)] when the polynucleotide is present in the renaturation process.[15,16,22] Our data showed that hydrogen bonds are formed between the glucose and the base moieties. Furthermore, other β-(1→3)-D-glucans, such as curdlan and lentinan, can form this same complex. Therefore, this complexation is characteristic for β-(1→3)-D-glucans.[23,24] Some other novel features for the complex include (1) the complex is remarkably stable (large binding constant) and considerably water-soluble in physiological conditions,[16] (2) the complexation occurs in a highly stoichiometric manner, and the stoichiometric number indicates that two schizophyllan units and three base units interact with each other [see Fig. 1(b)],[16] (3) when the s-SPG/DNA complex meets the corresponding complementary sequence, for example s-SPG/poly(dT) meets poly(dA), the complex dissociates immediately and hybridization takes place.[25]

When schizophyllan is used as an AS ODN carrier, the three features of (1)–(3) mentioned above are advantageous. This paper presents experimental evidence that the AS ODN in the complex executes its task more efficiently than that of naked

AS ODNs, using cutaneous melanoma cell lines and a phosphorothioate AS ODN to target the disruption of *c-myb* proto-oncogene.[26] *c-myb* is overexpressed in many melanoma cell lines and plays a central role in the exponential growth of this malignant neoplasm.[27,28] Depression of *c-myb* can be done using an AS ODN addressed to a targeted region of *c-myb* mRNA, the most effective antisense sequence of which is well-known.[28,29] Therefore, the *c-myb* antisense has been a good model assay system in antisense research.[29–31]

Results and Discussion

Complexation between Phosphorothioates and Schizophyllan.   Our previous studies[15,16,23,32] have shown that once s-SPG combines with phosphodiester oligonucleotides, the circular dichromic (CD) spectrum is drastically changed and a hypochromic effect is observed in the UV spectrum. These changes occur in the 200–400 nm wavelength range. Schizophyllan has no functional group to absorb the light in this range. On the other hand, the base moieties in nucleotides do have an absorbance. Therefore, the changes in CD and UV can be related to the conformational transition in polynucleotides, hence they can be used as a criterion for the complex formation. Figure 2(a) compares the CD spectrum of naked phosphorothioate AS-c-myb with that of a mixture of AS-c-myb and s-SPG (coded by AS-c-myb + s-SPG[33]) measured at 5 °C. The spectrum for AS-c-myb + s-SPG has two positive bands at 260 and 280 nm, while AS-c-myb has a broad band centered around 270 nm, indicating that the AS-c-myb/s-SPG complex is formed. Figure 2(b) presents the temperature dependence of the UV absorbance at $\lambda = 260$ nm for AS-c-myb itself and AS-c-myb + s-SPG at the same nucleotide molar concentration (20.2 μM, 6.67 μg/mL). At 5 °C, the absorbance of the mixture is lower than that of naked AS-c-myb because of hypochromism due to the complexation. With increasing temperature, the absorbance of naked AS-c-myb increases due to a decrease in the number of stacked bases. On the other hand, the absorbance for the mixture stays at 1.88 in the temperature range of 5–40 °C, then starts to increase with further increases in temperature above 50 °C, and finally merges into that of naked AS-c-myb above 75 °C. This behavior can be explained as follows. (1) In the range of 5 °C < T < 40 °C, the constant absorbance means that the complex conformation does not change upon heating, (2) at 40 °C, the complex starts to dissociate, therefore the absorbance increases. (3) T > 75 °C, the complex dissociates completely, thus the absorbance of the mixture is identical
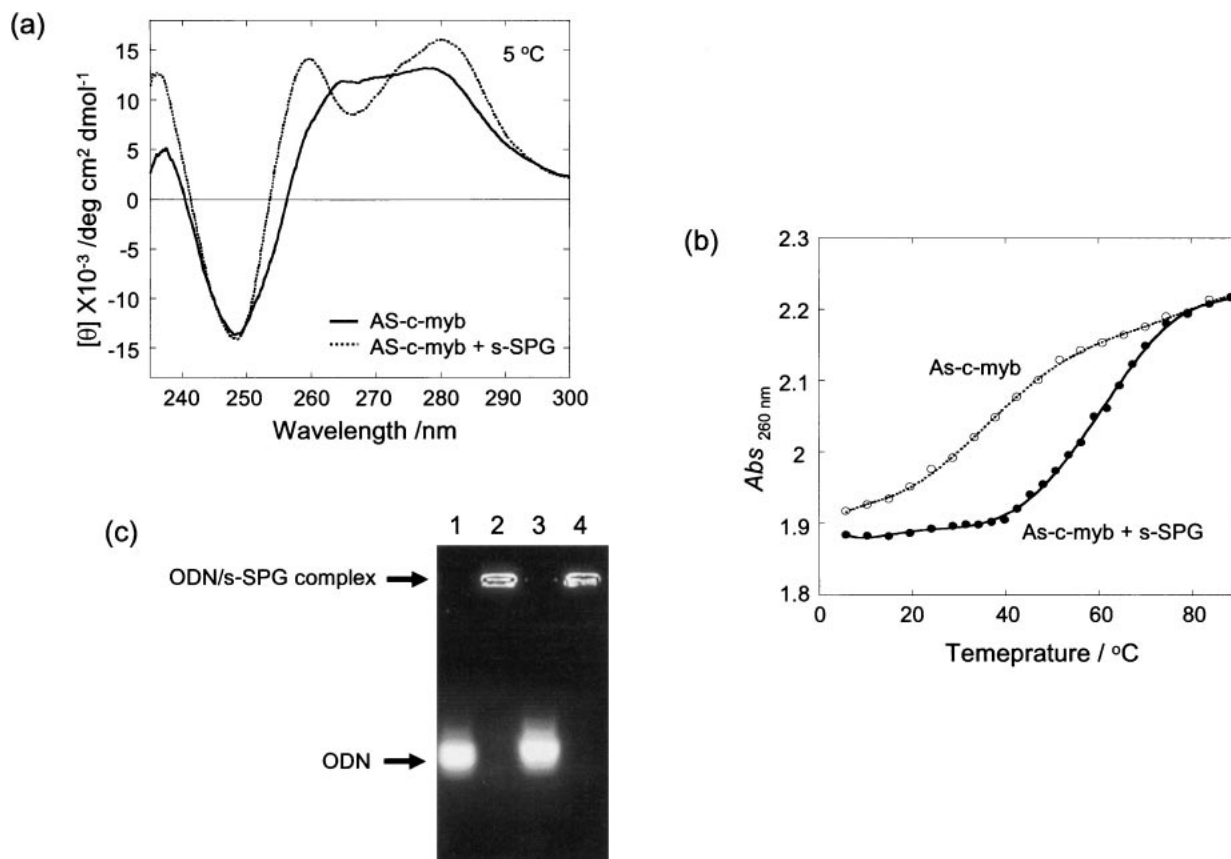
Fig. 2.   Confirmation of the complex formation between AS-c-myb and s-SPG complex: (a) The CD spectra of AS-c-myb and its mixture with s-SPG, (b) the temperature dependence of the UV absorbance, (c) comparison of the gel migration patterns between AS-c-myb and its mixture with s-SPG. In the panel (c), 2 wt % NuSieve agarose gel (BMA) was used and the gel was stained with GelStar[®] (BMA). Lane 1: AS-c-myb, lane 2: AS-c-myb + s-SPG, lane 3: S-c-myb, and lane 4: S-c-myb + s-SPG.

to that of naked AS-c-myb. The results presented in Fig. 2(b) indicate that the complex is stable at both room and cell-incubation (37 °C) temperatures.

Figure 2(c) compares the gel electrophoresis migration patterns between naked AS-c-myb and AS-c-myb + s-SPG (or naked S-c-myb and S-c-myb + s-SPG). Here, only the ODNs are stained by GelStar[®]. The ODNs themselves migrate, however, the mixture of the ODNs and s-SPG do not at all. This feature also provides evidence of complex formation in the mixture.[16,32]

Proliferation after Administrating AS OND and s-SPG. Figure 3 shows the relation between the number of cells (estimated by WST-8 assay) and the culture days after the melanoma A375 cells were treated with AS-c-myb, AS-c-myb/s-SPG, s-SPG, or saline (control). Here, the dose weight ratio of s-SPG:AS ODN was always fixed at 150 µg:50 µg, and the total doses for AS ODN, s-SPG, and AS ODN/s-SPG were 50, 150, and 200 µg/mL, respectively (i.e., the amount of the AS ODN dose was always kept at 50 µg). The number of the cells increased drastically (probably exponentially) when the cells were exposed to s-SPG and the saline reference, and the cell number was identical between s-SPG and the reference. This means that s-SPG itself does not have any cytostatic effect at this dose. When the cells were treated with AS-c-myb and AS-c-myb/s-SPG, the proliferation for both systems was reduced compared with the control. After 5 days, the number
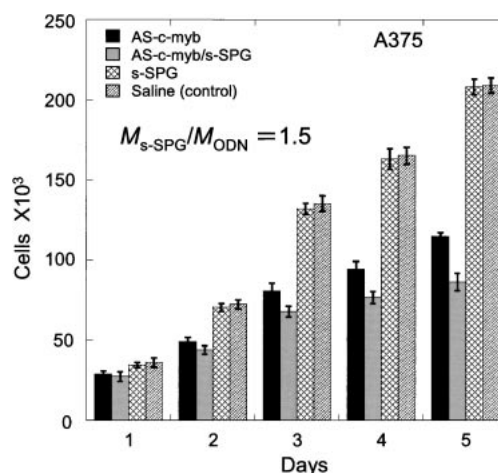

Fig. 3.   Comparison of the cell growth after treated melanoma A375 cells with AS-c-myb, AS-c-myb/s-SPG, and s-SPG, comparing with the control assay in which only saline was added. The cells were grown in the presence of 50 µg/mL of AS-c-myb, 200 µg/mL of AS-c-myb/s-SPG (containing 50 µg/mL AS-c-myb), and 150 µg/mL of s-SPG at 37 °C. Cell number was determined by WST-8 assay. The molar concentration ratio ($M_{s\text{-}SPG}/M_{poly(dA)}$) is fixed at 1.5.

of cells for these systems becomes almost half that of the control. Furthermore, AS-c-myb/s-SPG shows a lower proliferation than naked AS-c-myb itself at any time, suggesting that the antisense activity is more enhanced for the complex than for the naked ODN. From this experiment, three culture days are long enough to provide the distinguishable difference in the proliferation. Thus, hereinafter we will compare the proliferation only after three days and, for convenience, the number of cells is normalized by that of the control, and the resultant value was defined as the cell growth.

Figure 4 shows the relationship between cell growth and the s-SPG:AS-c-myb molar ratio. Here, the AS-c-myb dose is fixed at 50 μg/mL and only the s-SPG dose is changed from 0 to 1000 μg (corresponding to a molar ratio of 10, where the repeating unit molecular weights of AS-c-myb and s-SPG are 330 and 650, respectively). The cell growth decreases with an increasing ratio and reaches a minimum around the ratio = 1–2, then abruptly increases. As mentioned in the introduction, the complex consists of two s-SPG repeating units and three base units (see Fig. 1(b)), thus the stoichiometric ratio is 2/3.
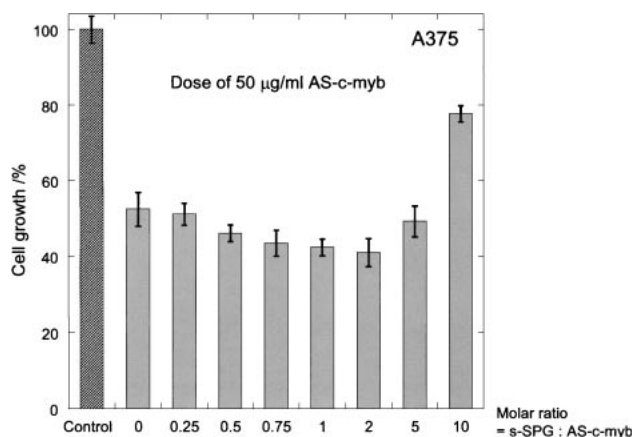
The molar ratio to give the minimum is larger than the stoichiometric ratio. Hereinafter we will evaluate the cell growth at the molar ratio = 1.5.

When the molar ratio was at 5 or 10, the AS-c-myb/s-SPG solution was found to be slightly opaque owing to precipitation of excess s-SPG. Therefore, the increase in the cell growth at the larger ratios can be explained as such that some AS-c-myb molecules co-precipitated with s-SPG, thus the effective AS-c-myb concentration in the solution decreased.

Figure 5 presents the s-SPG dose dependence of the cell growth for C32 and A375 cells (without AS ODN) over the s-SPG dose range from 18.75 to 600 μg/mL. The cell growth decreases slightly, by 10–7% at 600 μg/mL, indicating that there is a slight cytostatic-effect for s-SPG when excess is added. When we examined the antisense effect in this work, most experiments were carried out in an s-SPG dose range of less than 300 μg/mL, an amount of s-SPG that reduces cell growth by no more than 5%. On the other hand, the AS ODN reduces the cell growth by about 50% (see Fig. 4). Therefore, the cytotoxicity of s-SPG is negligible when we discuss the antisense effect.

Dose Dependence of Proliferation.   Figure 6 shows the

Fig. 4.   Relation between the cell growth and s-SPG:AS-c-myb molar ratio for A375. The AS-c-myb dose was fixed at 50 μg/mL.
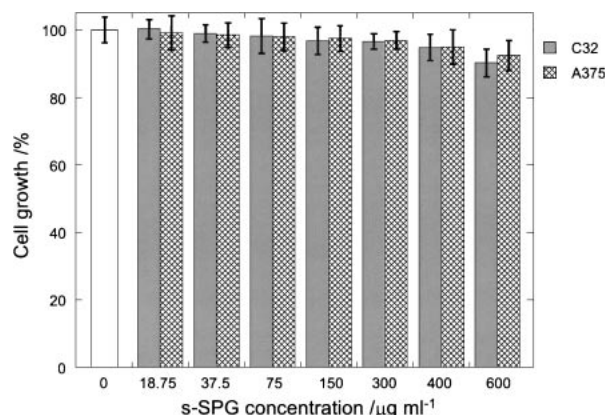
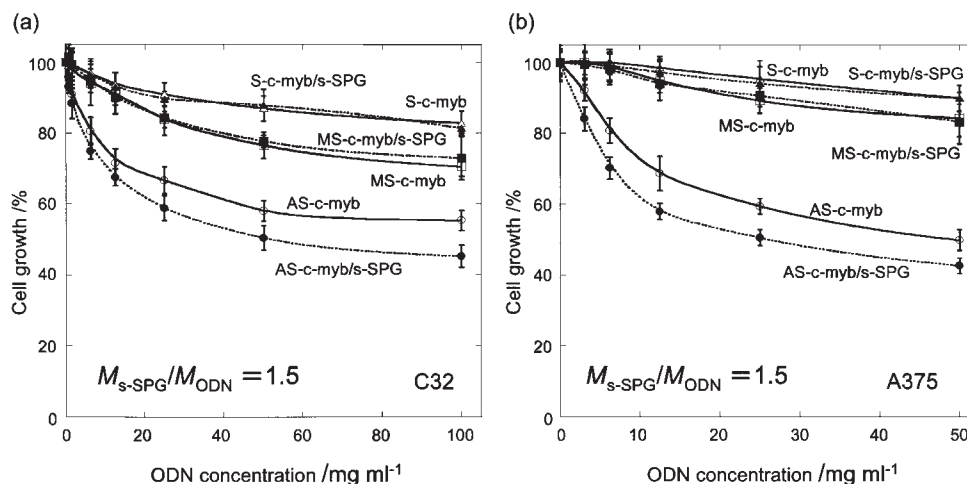Fig. 5.   Cellular toxicity for schizophyllan dose for C32 and A375 cells.

Fig. 6.   Dose dependence of the melanoma cell growth for C32 (a) and A375 (b), when treated with AS-c-myb (○), S-c-myb (△), MS-c-myb (□), AS-c-myb/s-SPG (●), S-c-myb/s-SPG (▲), and MS-c-myb/s-SPG (■). The cell number was determined after 3 days. The molar concentration ratio ($M_{s\text{-SPG}}/M_{poly(dA)}$) is fixed at 1.5.

Table 1. Statistical Analysis of the Antisense Test for the Cell Growth at 100 mg/mL Dose for C32

	S-c-myb	S-c-myb/s-SPG	MS-c-myb	MS-c-myb/s-SPG	AS-c-myb	AS-c-myb/s-SPG
Average	82.7	83.0	73.0	72.6	56.4	45.0
Dispersion	10.9	16.6	10.0	28.7	4.3	8.5
n	4	4	5	5	5	5
T-value		0.47		0.21		6.5

ODN dose dependence of the cell growth for the C32 (Panel a) and A375 (Panel b) cells. Here, the ODN dose was controlled by changing the ODN concentration added to the plate. To confirm the antisense effect, the sense sequence (S-c-myb), its complex (S-c-myb/s-SPG), the mismatch sequence (MS-c-myb), and its complex (MS-c-myb/s-SPG) were examined as well as AS-c-myb and AS-c-myb/s-SPG. For each 100 µg/ mL dose, we calculated the dispersion and T-value, summarized in Table 1. As shown by the figure, the general trends for both cell lines are consistent with each other. AS-c-myb and AS-c-myb/s-SPG reduce the cell growth with increasing ODN concentration and growth decreases 45–50% at the highest dose for both lines. Although both (AS-c-myb and AS-c-myb/s-SPG) reduce the growth rate drastically, it is clear that AS-c-myb/s-SPG reduces the cell growth more efficiently than naked AS-c-myb. As presented in Table 1, the T-value between AS-c-myb and AS-c-myb/s-SPG is much larger than the others, indicating that the complex with AS-c-myb causes a statistically significant difference. On the other hand, S-c-myb and S-c-myb/s-SPG show a relatively small reduction and the growth decreases to only 85–90% at the highest dose. Furthermore, the growth between S-c-myb and S-c-myb/s-SPG is similar at the same ODN concentration, confirming the negligibly small cytotoxicity for s-SPG. Even the sense sequence reduces the growth. According to the previous work, phosphorothioate DNAs tend to depress cells growth in a nonspecific manner.[4,34–37] Therefore, this simple cytotoxicity effect should be the main reason for the decrease in cell growth for S-c-myb and S-c-myb/s-SPG. When we exposed both cells to the G-rich mismatch sequence (MS-c-myb and MS-c-myb/s-SPG), the growths were lowered more than those corresponding to the sense sequence (i.e, S-c-myb and S-c-myb/s-SPG). This can be explained by anti-proliferation activity for the contiguous G residues.[38] Figure 6 demonstrates that administering AS-c-myb decreases proliferation, and this effect is beyond both the cytotoxicity of the phosphorothioates and the non-antisense effect of the G-rich sequence. The most important result in this experiment is that the complex always provides a more enhanced anti-proliferation effect than naked AS ODN.

Although the data are not shown, when we carried out the same experiment as shown in Fig. 6(b) using the phosphodiester AS-c-myb (instead of phosphorothioate), the antisense effect was not as clearly observed in comparison with the phosphorothioate system. The poor antisense effect was not improved much even when we used the corresponding complex. The growth was 70–80% at a 50 µg/mL dose for the A375 cells. These features may be ascribed to the fact that phosphorothioate is taken up by cells much easier than phosphodiester, because the surface protein responsible for ODN up-take has a higher affinity for phosphorothioate than for phosphodiester.[4,7,39,40]
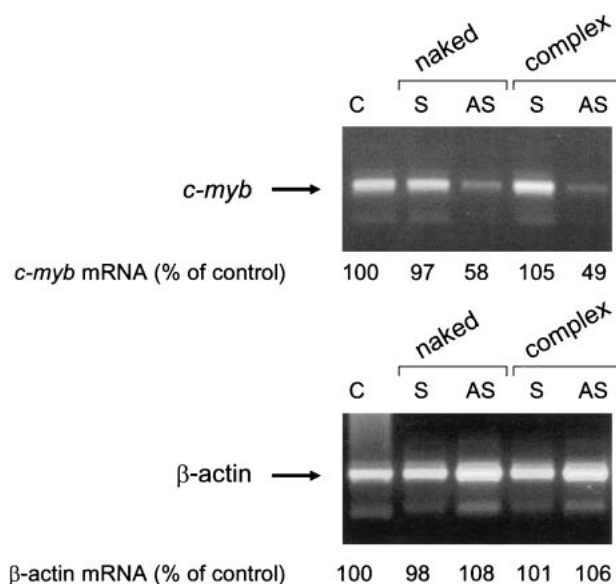

Fig. 7.   Expression of β-actin and *c-myb* mRNA determined by RT-PCR analyses. A375 cells were cultured in 24-well plates at initial concentration of 2×10^4 cells/mL in medium, and were treated with naked ODNs (60 µg/mL) and ODNs/s-SPG (containing 60 µg/mL AS-c-myb) for 2 days before extraction of RNA. Extracted RNA was subjected to RT-PCR and amplified DNA fragments were run on 1.5% (w/v) agarose gels. Lane C: untreated cells (control), lane S: S-c-myb or S-c-myb/s-SPG, lane AS: AS-c-myb or AS-c-myb/s-SPG. Amplified DNA fragments were quantified by Alpha DigiDOC gel documentation system.

Confirmation of the Antisense Effect by Reverse-Transcriptase-Mediated PCR. The enhanced anti-proliferation effect for AS-c-myb/s-SPG in Fig. 6 strongly supports that the antisense ODN depresses *c-myb* expression. Encouraged by these results, we examined whether naked AS-c-myb and its complex carried out their task well in eliminating the target mRNA in a sequence-specific manner using RT-PCR. The results are presented in Fig. 7. First of all, we evaluated the amount of β-actin mRNA, comparing the control (no administration of ODN) with the administration of S-c-myb, S-c-myb/ s-SPG, AS-c-myb, and AS-c-myb/s-SPG. As shown in the lower panel in Fig. 7, there is no difference among the samples, indicating that the administration of these ODNs does not have any effect on the amount of β-actin mRNA. However, when we isolated the *c-myb* mRNA and evaluated the amount of mRNA by the same manner (the upper panel in the figure), the AS-c-myb and AS-c-myb/s-SPG did decrease the amount of mRNA, while both AS-c-myb and AS-c-myb/s-SPG did not. This result indicates that antisense ODN eliminated the target mRNA in a sequence-specific manner. Moreover, the com-
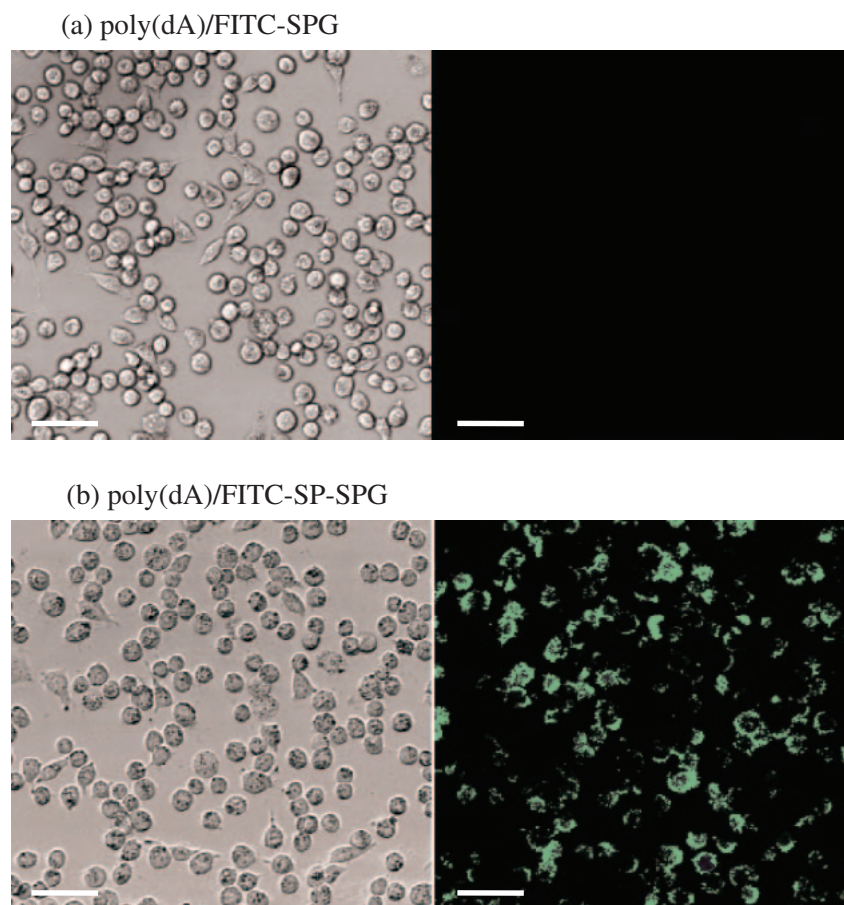
(a) poly(dA)/FITC-SPG

(b) poly(dA)/FITC-SP-SPG

Fig. 8.   Confocal fluorescent microscopy images when A375 cells were treated with poly(dA)/FITC-SPG complex (a) and poly(dA)/ FITC-SP-SPG (b). Phase contrast images of the same field of cells are shown left each fluorescence image. The bar indicates 100 µm.

plex reduces the amount more drastically than the naked one, confirming the antisense effect is enhanced in the complex.

Up-Take of s-SPG and ODN.   One of the possible ways to explain the enhanced antisense effect of the complex is that schizophyllan may increase the up-take efficiency. To examine whether s-SPG is taken into a cell, we exposed A375 cells to a complex made from poly(dA) and FITC-SPG (fluorescein iso-thiocyanate-labeled schizophyllan) and observed the cell mor-phology with confocal fluorescent microscopy. Figure 8(a) shows a typical image obtained in this experiment. For compar-ison, we exposed the same cells to a complex made from poly(dA) and FITC-SP-SPG[41] (FITC-labeled, and spermine-appended schizophyllan) which was already observed to have an increase in the cellular uptake due to the positive charge, as seen in the confocal microscopic image in Fig. 8(b). Figure 8(b) clearly shows that the labeled materials are taken into a cytosol. On the other hand, there is no such a feature observed in Fig. 8(a). Therefore, we conclude that schizophyllan itself has no capability to induce endocytosis.

Nuclease-Mediated Hydrolysis of the Antisense ODNs. Figure 9 plots ΔAbs against the incubation time during the hy-drolysis by nuclease P_1 for AS-c-myb [AS-c-myb] and the complex made of s-SPG and AS-c-myb [AS-c-myb/s-SPG]. The nuclease P_1 effectively hydrolyzes single stranded DNAs and produces 5′-monophosphoric nucleic acids, resulting in in-
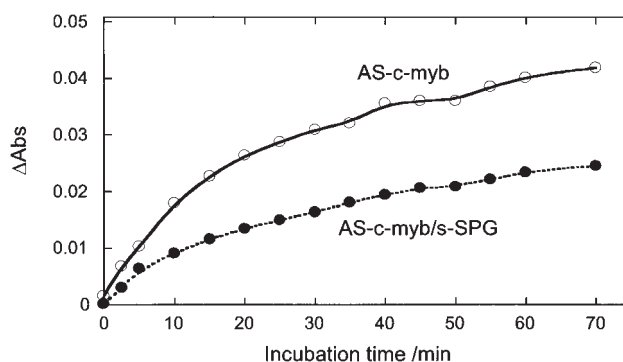

Fig. 9.   Increment of the absorbance during the hydrolysis by nuclease P_1 at 37 °C, comparing phosphodiester AS-c-myb [AS-c-myb (PO)], phosphorothioate AS-c-myb [AS-c-myb], and the complex made of s-SPG and phos-phorothioate AS-c-myb [AS-c-myb/s-SPG]. 15.2 µM of AS-c-myb was added to 2 mL of 3 mM sodium acetate (pH 6.0), and 1 mM ZnSO$_4$. The concentration of nuclease P_1 was 40 U/mL.

creasing ΔAbs.[42,43] Figure 9 shows that the complexation de-creases the ΔAbs change more effectively than the naked sam-ples, confirming our previous conclusion that the complex can protect the bound DNA form a nuclease attack.[44]
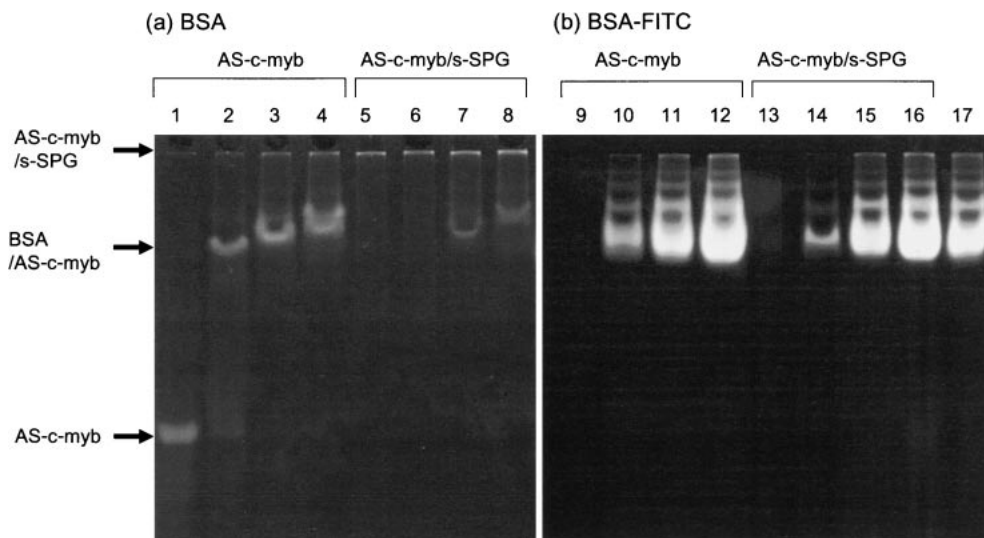
Fig. 10. AS-c-myb/s-SPG complex stability in the presence of BSA determined by gel shift assay. (a) BSA was mixed with AS-c-myb or AS-c-myb/s-SPG complexes. These mixtures were incubated for 30 min at 37 °C, and run on a 7.5% non-denaturing poly-acrylamide gel. The gel is stained by GelStar®, and the image was obtained by a gel documentation system (Alpha DigiDOC gel documentation system, Alpha Innotech). Lane 1–4: naked AS-c-myb (0.2 µg), lane 5–8: AS-c-myb/s-SPG (containing 0.2 µg AS-c-myb). Lanes 2 and 6: added 10 µg BSA, lanes 3 and 7: 50 µg BSA, and lanes 4 and 8: 100 µg BSA. (b) BSA-FITC was mixed with AS-c-myb (9–12) or AS-c-myb/s-SPG complexes (13–16). The gel is not stained by GelStar(r). Lane 9: AS-c-myb itself (0.2 µg), no BSA-FITC, lane 13: AS-c-myb/s-SPG (containing 0.2 µg AS-c-myb), no BSA-FITC, lanes 10 and 14: added 10 µg BSA-FITC, lanes 11 and 15: 50 µg BSA-FITC, and lanes 12 and 16: 100 µg BSA, lane 17: 50 µg BSA-FITC itself.

Stability of the Complex in the Cell-Culture Medium. Figure 10(a) compares the gel-shift between naked AS-c-myb and AS-c-myb/s-SPG in the presence of BSA. In the panel (a), the gel was stained with GelStar®, thus only nucleonic bases can be observed. In the absence of BSA, the naked AS-c-myb (0.2 µg) migrates a great distanse (lane 1). When we added 10 µg of BSA to 0.2 µg of AS-c-myb (lane 2, BSA concentration is about 1 mg/mL), there was still a small amount of the free AS-c-myb. However, most of them were incorporated into the BSA/AS-c-myb complex. When we added extra BSA (50 and 100 µg in lanes 3 and 4, respectively, i.e., BSA concentrations were about 5 and 10 mg/mL), there was no free AS-c-myb, and all ODN were bound to BSA. When we carried out a similar experiment for the AS-c-myb/s-SPG complex (0.2 µg of AS-c-myb and 0.6 µg of s-SPG), the addition of BSA provided essentially no change in migration. Lane 5 shows the complex itself (i.e., without BSA), stays at the starting hole.[16,32] When we added 10 µg of BSA to 0.2 µg of AS-c-myb (lane 6), the migration pattern was exactly the same as that of the complex itself. When we increased the BSA amount to excess, some AS-c-myb seemed to be taken out of the complex and bound to BSA, however, this amount is relatively small compared with that in the complex. Although the data are not shown, after we mixed the complex with serum and waited a few days, we obtained the same results as those in Fig. 10(a).

We carried out the same experiments as those in Fig. 10(a) using fluorescein-labeled BSA (BSA-FITC), where only BSA was observed. Figure 10(b) shows the results of the comparison between naked AS-c-myb (lanes 9–12) and the AS-c-myb/s-SPG complex (lanes 13–16). BSA-FITC was added at 0, 10, 50, and 100 µg for lanes 9 and 13, 10 and 14, 11 and 15, and 12 and 16, respectively. In lane 17, only BSA-FITC (50 µg) was loaded. As shown in the figure, there is no difference in

the migration pattern between the naked ODN and the complex. This feature indicates that the complex is not bound to BSA, otherwise the BSA would be in the staring hole.

Based on the above results, we conclude that AS-c-myb/s-SPG is considerably stable in a solution containing an extra amount of BSA, and that the complex remains intact in the presence of BSA. On the other hand, naked AS-c-myb is bound to BSA. The BSA concentration range examined here covers that for the cell-culture medium. Therefore, the complexed AS-c-myb hardly bound to BSA when we cultured the cells.

A Possible Mechanism for the Enhanced Antisense Effect for the Complex. Our culture medium contains an albumin, BSA, a necessary ingredient for growth of the cells, as well as some nuclease. It is well-known that BSA itself easily binds to hydrophobic molecules. In particular, phosphorothioate is more hydrophobic than phosphorodiesters,[45–47] so BSA should bind to phosphorothioate strongly. In fact, Fig. 10(a) shows the strong affinity between the naked ODN and BSA. For human leukemia cells, there is evidence that AS ODN is up-taken by receptor-mediated endocytosis relating to the albumin recognition site on the cell surface.[45] If the albumin mediated endocytosis was the major route for the up-take of AS-c-myb in our system, it is difficult to explain the enhanced antisense effect of the complex, because the AS-c-myb in the complex can not bind to BSA.

It is surprising that the cellular up-take of phosphorothioate ODNs occurs to a greater extent than would be expected on the basis of charge and size consideration. In fact, the phosphodiester AS ODN shows a poor antisense effect in our system. The pharmacokinetics of phosphorothioate ODNs, especially its cellular up-take mechanism, are not well understood. However, it has been suggested that the up-take of phosphorothioate AS ODNs can be mediated by pinocytosis or adsorptive endo-

cytosis facilitated by proteoglycans and the binding proteins in the cell membrane.[4,39,40] Therefore, it seems that the presence of AS ODN free from BSA is important, and since the complex can prevent the AS ODN from binding to BSA, the antisense effect was enhanced in our system. Furthermore, the complex can protect the bound AS ODN from nuclease attack. In summary, we can conclude that the antisense effect is enhanced in the complex by protecting AS ODN from unfavorable interactions (such as hydrolysis or absorption) with proteins in the culture medium.

Conclusion

The present report describes the formation of a phosphorothioate–oligonucleotide complex with s-SPG in the same manner as phosphodiester oligonucleotides. Furthermore, we carried out an in vitro antisense assay combining melanoma cell lines and a phosphorothioate antisense oligonucleotide (AS ODN) to depress *c-myb* mRNA, and found that the AS ODN bound in the complex reduces cell growth more efficiently than that of naked AS ODN. The reason for the enhanced depression has been rationalized by a new proposal that states the complex can prevent the AS ODN from binding to BSA in the culture medium as well as protecting AS ODN from nuclease. The present results show, therefore, that schizophyllan can be used as an AS ODN carrier.

Experimental

Materials.   Taito Co. Ltd., (Japan) kindly supplied the schizophyllan sample. The weight-average molecular weight (M_w) and the number of repeating units were found to be 1.5×10^5 and 231, respectively.[16,17] The oligonucleotide (ODN) sequence of 5′-GTG CCG GGG TCT TCG GGC-3′ is well known to bind to *c-myb* mRNA and lead to the depression of *c-myb*.[26,28–31] However, since short and hetero ONDs cannot bind with s-SPG,[16,32] we had to attach a poly(dA) tail with 40 bases at the 3′ end of this sequence. Thus, in this study, 5′-GTG CCG GGG TCT TCG GGC-(dA)$_{40}$-3′ phosphorothioate was used as an AS ODN and is denoted by AS-c-myb, hereafter. To examine whether the antisense effect really strives to suppress the cell growth, we used an ODN containing the sense sequence 5′-GCC CGA AGA CCC CGG CAC-3′[28,29] as a reference. Additionally, the antisense phosphorothioate ODN can show anti-proliferation activity through a non-antisense path when the sequence contains some contiguous G residues.[38] Therefore, as a second control, a mismatch bases of AS-c-myb; 5′-GTC CTG GGG TCG TCG GGC-3′[30] was used (the mismatched sequences are underlined). For the same reason as those in the antisense sequence, the sense and mismatched sequences were connected to the (dA)$_{40}$ tails at the 3′ ends and denoted by S-c-myb and MS-c-myb, respectively. AS-c-myb, S-c-myb, and MS-c-myb were synthesized at Hokkaido System Science (Hokkaido, Japan) and purified with high-pressure liquid chromatography.

MEM® (minimum essential medium), nonessential amino acids, fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Gibco/BRL. Dulbecco's modified Eagle's medium (DMEM®) was obtained from Nissui Pharmaceutical Co. Ltd. Bovine serum albumin (BSA) was obtained from Sigma. Fluorescein isothiocyanate (FITC) was purchased from Dojin, Japan. An ultrafiltration device (Centricon® plus-10; NMWL, 10000) was purchased from Millipore (Bedford, MA). Poly(dA) was purchased from Amasham (Kyoto, Japan).

Fluorescein-appended BSA (BSA-FITC) was synthesized by mixing a BSA (109 mg, 1.7 μmol)/water solution with a fluorescein-4-isothiocyanate (0.7 mg, 1.8 μmol)/acetone solution (Doujin, Japan). The mixture was purified by dialysis with distilled water several times and then lyophilized. Spermine-appended SPG (SP-SPG) was synthesized as described in our previous paper.[41] FITC-labeled schizophyllan (FITC-SPG) and FITC-labeled SP-SPG (FITC-SP-SPG) were prepared as follows. Schizophyllan (60 mg) was dissolved in purified DMSO (2 mL). To this solution was added FITC (1 mg), and the mixture was vigorously stirred in the dark at room temperature for 60 h. The resulting derivative was purified by ultrafiltration with purified DMSO (3 mL) to remove excess FITC. The solutions were lyophilized to obtain FITC-SPG and FITC-SP-SPG.

Preparation of Complex between ODN and s-SPG.   2 mg of S-c-myb, MS-c-myb, and AS-c-myb were dissolved in 1 mL of 10 mM Tris buffer solution (pH = 7.8). An appropriate concentration of s-SPG/DMSO solution was added to the ODN solution so that the water volume fraction was always adjusted to 0.9 after mixing. The molar ratio ($M_{s\text{-SPG}}/M_{ODN}$) was controlled at 1.5, except when we measured the $M_{s\text{-SPG}}/M_{ODN}$ dependence of the cell growth (Fig. 4), where $M_{s\text{-SPG}}$ and M_{ODN} are the molar concentrations for the s-SPG and ODNs repeating units, respectively. After the ODN and s-SPG mixture was kept at 5 °C for 1 night to lead the complexation, DMSO was removed by ultrafiltration. After the filtration, the final concentration of ODN was determined by measuring the ultraviolet absorbance. To confirm the complexation between ODNs and s-SPG, we measured the circular dichroism (CD) spectrum, ultraviolet (UV) spectrum, and gel electrophoresis migration pattern. The details for these experiments are described in the preceding paper in this series of work.[15,16,23–25,32]

Cell Culture and Confocal Fluorescent Microscopy.   The melanoma cell lines C32 and A375 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The C32 cells were maintained in MEM® supplemented with 0.1 mM nonessential amino acids and 10% FBS. The A375 cells were maintained in DMEM® supplemented with 10% FBS. All medium contained a 1 wt % penicillin/streptomycin mixture. Cell incubation was always carried out at 37 °C in fully humidified air containing 5 wt % CO_2.

A375 cells (2×10^5 cells) were grown just before confluence in a 96-well tissue culture plate (NUNC). The FITC-SPG–poly(dA) complex and FITC-SP-SPG–poly(dA) complex [the molar ratio ($M_{s\text{-SPG}}/M_{poly(dA)}$) was controlled to be 1.5] were added to the medium (final concentration 200 μg/mL). The cells were incubated for 12 h at 37 °C in a 5% CO_2 incubator. The cells were then washed three times with PBS (100 μL) and examined by fluorescence microscopy. Images of the samples were collected by fluorescence microscopy on an ECLIPSE TE2000-U (Nikon, Tokyo, Japan) attached to a confocal scan unit Radiance 2100C (Bio-Rad, Tokyo, Japan) using a 20× objective lens (the total magnification was 200×).

Measurement of Cell Proliferation.   Cells were seeded in 96-well plates (NUNC) at a density of 2×10^4 cells/mL (1 well/100 μL) and allowed to attach to the plate overnight. The following day, the medium (MEM® supplemented with 0.1 mM nonessential amino acids and 10% FBS) was changed to fresh medium, and cells were treated with an appropriate amount of ODN (0.39–100 μg/mL), ODN/s-SPG complex (containing 0.39–100 μg/mL ODN), or s-SPG (1–200 μg/mL). Subsequently, cells were incubated for 1, 2, 3, 4, or 5 days before measurement of cell growth. The cell number was evaluated by use of a Cell Counting Kit-8® (Dojindo,

Japan) called the WST-8 assay. The WST-8 assay uses a novel tetrazolium salt 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt (WST-8), which produces a water-soluble formazane dye upon reduction mediated by dehydrogenase in living cells.[47] After incubation for 1, 2, 3, 4, or 5 days, 10 µL of the Cell Counting Kit-8 working solution (containing WST-8 and 1-methoxy-5-methylphenazinium methosulfate) was added to each well, followed by incubation for 4 h at 37 °C. Plates were read on a microplate reader Multiskan JX (Thermo Labsystems) using a wavelength of 450 nm in comparison with 650 nm. Each set of control or treated cells was tested in triplicate wells, and the mean and standard deviation of the values was plotted. All growth studies were carried out at least twice.

Nuclease-Mediated Hydrolysis. We measured the increment in the ultraviolet absorbance (ΔAbs) at 260 nm during the hydrolysis of the nucleotides with a JASCO V-570. The hydrolysis of AS-c-myb by nuclease P_1 (from *Penicillium citrium*, Sigma)[42,43] was carried out at 37 °C in a reaction mixture in which 15.2 µM of AS-c-myb was added to 2 mL of 3 mM sodium acetate (pH 6.0) and 1 mM $ZnSO_4$. The concentration of nuclease P_1 was 40 U/mL.

Isolation of Total RNA and Reverse-Transcriptase-Mediated PCR. To confirm that the reduced proliferation was due to decreasing *c-myb* expression by the antisense effect, the expression of *c-myb* was evaluated at the mRNA level by the reverse-transcriptase-mediated PCR (RT-PCR) method.[48–50] A375 cells were plated in 24-well plates (NUNC) at an initial concentration of 2×10^4 cells/mL in the medium and allowed to attach to the plate overnight. The following day, the cells were treated with 60 µg/mL ODN and ODN/s-SPG complex. After 2 days, the cells were harvested with 0.25% trypsin-EDTA in PBS. The total RNA was isolated with a RNeasy Mini Kit (QIAGEN) as recommended by the manufacturer. RT-PCR was performed with a ThermoScript RT-PCR system (Invtrogen). A PCR tube contained RNA (0.9 µg), oligo (dT) primer, dNTPs (10 mM, 2 µL), DTT (0.1 M, 1 µL), and RNaseOUT (2 U/µL, Thermoscript RT (0.75 U/µL). Synthesis of the first-strand cDNA was done at 55 °C for 40 min in a DNA thermal cycler (T gradient, Biometra, Germany). The resulting cDNA fragments were amplified with 2 U/ml of *Taq* polymerase, $MgCl_2$ (50 mM, 1.6 µL), dNTPs (10 µM, 1 µL), and 15 pmol of each primer. Regents were from Invitrogen. Thermal cycling consisted of 1 min denaturing at 95 °C, 1 min annealing at 55 °C (*c-myb*) or 57 °C (β-actin), and 1 min of extension at 72 °C. This was repeated for 40 cycles (*c-myb*) and 28 cycles (β-actin), with a final extension period of 3 min. Primer sequences were as follows: *c-myb*, 5′-AAT TAA ATA CGG TCC CCT GAA-3′ (forward), 5′-TGC TCC TCC ATC TTT CCA CAG-3′ (reverse), 423 bp predicted product size. β-actin, 5′-GGC TAC AGC TTC ACC ACC AC-3′ (forward), 5′-AGG GCA GTG ATC TCC TTC TG-3′ (reverse), 370 bp predicted product size. PCT products were resolved by agarose gel electrophoresis and visualized by GelStar®. Amplified DNA fragments were quantified by a gel documentation system (Alpha DigiDOC gel documentation system, Alpha Innotech).

References

  #   This paper is the 13th paper in the series of polysaccharide–polynucleotide complexes.

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