An Antisense Oligonucleotide Complementary to a Sequence in Iγ2b Increases γ2b Germline Transcripts, Stimulates B Cell DNA Synthesis, and Inhibits Immunoglobulin Secretion

By Toshio Tanaka, Charles C. Chu, and William E. Paul

From the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892

Summary

An antisense phosphorothioate (S)-oligonucleotide to a sequence in the intervening (I) region of the γ2b immunoglobulin (Ig) heavy chain gene inhibits Ig secretion by B cells stimulated with lipopolysaccharide (LPS) or LPS plus interleukin 4. It is also a striking stimulant of DNA synthesis by resting B cells. The antisense S-oligonucleotide causes a 10–20-fold increase in the expression of the γ2b germline transcript. Among mutants of the antisense S-oligonucleotide, some show all the effects whereas others are inactive. A similar hierarchy exists in the quantitative biological activities of mutant S-oligonucleotides and in their capacity to hybridize to the sense oligonucleotide, strongly suggesting that an Iγ2b sequence in the RNA transcript or in the noncoding strand of the DNA is the target of the antisense S-oligonucleotide. The possible relationship of the overexpression of the germline γ2b transcript to the biological functions of the Iγ2b antisense S-oligonucleotide is discussed.

Stimulants of Ig class switching in normal B cells and in B lymphomas cause the appearance of RNA transcripts derived from the exons that compose the constant (C) region of the Ig heavy (H) chain that will be expressed by those cells when they have switched, spliced to sequences derived from an exon located 5′ to the switch region for that same H chain class (1–4). These transcripts are referred to as germline transcripts and the exon specifying the RNA segment that is spliced to the C_H gene is designated I_H (3, 4). The first such germline transcript to be characterized was that for γ2b (4). It is composed of Iγ2b spliced to Cγ2b and is induced by stimulating Abelson murine leukemia virus–transformed pre-B cells or normal B cells with LPS. Similar germline transcripts have been reported for γ1 (5–7), γ3 (8), ϵ (9–12), and α (13, 14). In general, specific stimuli that lead to switching cause the appearance of the homologous germline transcript before the actual switch event (for review, see references 15 and 16). It has been suggested that transcription of a given I region is critical to the recombination events that result in switching, and that the switch-inducing specificity of stimulants such as IL-4 resides in their induction of transcription of the relevant I region(s).

An alternative proposal is that the I_H/C_H germline transcript might have some function, either through a product it may encode or through some direct action of the RNA. In the experiments described here, we attempted to test this alternative hypothesis by using both conventional and phosphorothioate (S)[1] oligonucleotides that were antisense to a specific sequence within Iγ2b in an effort to diminish Iγ2b/Cγ2b RNA levels. To our surprise, one such phosphorothioate Iγ2b antisense oligonucleotide, and some variants of it, proved to be potent inhibitors of the secretion of all classes of Ig, powerful stimulants of B cell DNA synthesis, and led to striking increases, rather than the expected decrease, in steady-state levels of Iγ2b/Cγ2b RNA. Evidence is presented to suggest that the biological activity of these phosphorothioate oligonucleotides is indeed due to their capacity to hybridize to Iγ2b.

Materials and Methods

Animals. Virus-free BALB/c female mice, 8–12 wk of age, were obtained from Division of Cancer Therapy Animal Program, National Cancer Institute, Bethesda, MD.

Culture Medium. RPMI 1640 (Biofluids Inc., Rockville, MD) supplemented with 10% FCS (HyClone Laboratories, Logan, UT), L-glutamine (2 mM), 2-ME (0.05 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) was used as culture medium.

Reagents. Percoll was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). LPS W extract from *Escherchia coli* 0111:B4 was

[1] *Abbreviations used in this paper:* AS, an oligonucleotide "antisense" to positions 160–177 in the Iγ2b exon; S, phosphorothioate; SE, an oligonucleotide "sense" to positions 160–177 in the Iγ2b exon.

obtained from Difco Laboratories, Inc. (Detroit, MI) and was used at 20 μg/ml in all experiments. Mouse rIL-4 was obtained from a baculovirus (AcMNPV.IL-4) prepared by Cynthia Watson (Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, Bethesda, MD). O-oligonucleotides were prepared using 380B DNA synthesizer (Applied Biosystems, Foster City, CA). Several S-oligonucleotides, including four independent preparations of an oligonucleotide "antisense" to positions 160–177 in the Iγ2b exon (AS), were obtained from Synthecell (Rockville, MD) and from Genosys Biotechnologies, Inc. (The Woodlands, TX). Anti-IgM antibody was obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL) and Dextran–anti-IgD conjugate ($H\delta^a/1$) was kindly provided by Dr. James Mond (Uniformed Services University of the Health Sciences) (17).

Preparations of B Cells. Cell suspensions were prepared from spleens. Red blood cells were lysed with ammonium-chloride potassium carbonate buffer. Nucleated cells were washed twice in HBSS and incubated at 4°C for 1 h with anti-Thy-1.2 (30H12) (18), anti-CD4 (Gk1.5) (19), and anti-CD8 (2.43) (20) antibodies. After washing, T cells were removed by complement-mediated cytotoxicity. Small B cells were separated by discontinuous Percoll-gradient centrifugation (70, 66, 60, and 50%). The cells at the interphase between 66 and 70% were collected and used as resting B cells. This procedure routinely yielded cells that were >85% surface IgM positive and <1% CD3 positive. Lymph node B cells were prepared by a magnetic bead purification using a mixture of fluoresceinated (Fl) anti-CD3, -CD4, -CD8, and -Thy-1.2 (21), yielding a population that was >95% surface IgM and B220 positive.

Measurement of Ig Concentration in Culture Supernatant. Purified B cells were cultured in 96-well flat-bottomed plates (Costar Data Packaging Corp., Cambridge, MA) with LPS or LPS plus IL-4 in the absence or presence of various concentrations of S-oligos for 6 d. Supernatants were harvested and Ig concentration in the supernatants was measured by an ELISA using Immunolon 2 plates (Dynatech Laboratories, Inc., Alexandria, VA) that has been described in detail elsewhere (22). Briefly, a fluorescent product, generated from cleavage of 4-methyl umbilliferyl phosphate by bound alkaline phosphatase-conjugated antibodies, was quantitated on a fluorometer (Allergenetics, a division of Axonics, Inc., Mountain View, CA). Fluorescence units were converted to Ig concentration from standard curves, determined using purified myeloma proteins of known concentration.

Measurement of [^{3}H]thymidine Incorporation and Cell Number. Cells were pulsed with 1 μCi [^{3}H]thymidine (ICN Radiochemicals, Irvine, CA) for the last 6 h and radioactivity was measured in a liquid scintillation spectrometer. Total viable cell number was assessed in a hemocytometer using trypan blue dye exclusion to distinguish viable cell number.

Detection of Surface Ig-positive Cells. B cells were incubated with LPS or LPS plus IL-4 in the absence or presence of S-oligos (0.5 μM) for 5 d. Stimulated B cells were treated with acid-buffer (pH 3.0) in order to remove Ig attached through Fcγ receptors. After washing, cells were stained with appropriate Fl-goat anti-mouse Ig for 1 h at 4°C. Surface Ig-positive cells were determined by cytometric analysis on a FACScan® (Becton Dickinson and Co., Mountain View, CA), using PI gating to limit analyses to living cells.

SDS-PAGE Analysis of Synthesized Proteins. B cells were stimulated with LPS, LPS plus AS (0.5 μM), LPS plus SE (0.5 μM), or LPS plus IL-4 for 5 d. Viable cells were obtained by Ficoll-Hypaque centrifugation. After washing, cells were incubated with methionine-free medium containing 2% FCS for 1 h at 37°C. Subsequently, cells were incubated in fresh methionine-free medium in the presence of 500 μCi/ml of ^{35}S-methionine (2,800 Ci/mmol; Amersham Corp., Arlington Heights, IL) for 2 h at 37°C and incubated with culture medium for another 3 h. Culture supernatants were harvested. Cells were washed with ice cold PBS and 1 mg/ml glucose, and lysed by NP-40 buffer. Culture supernatants and cell lysates were further treated with 10% TCA and precipitable fractions were obtained. Radioactivity of the samples was measured and constant amounts of TCA-precipitable radioactivity from culture supernatants and cell lysates were loaded on SDS-PAGE under reducing conditions.

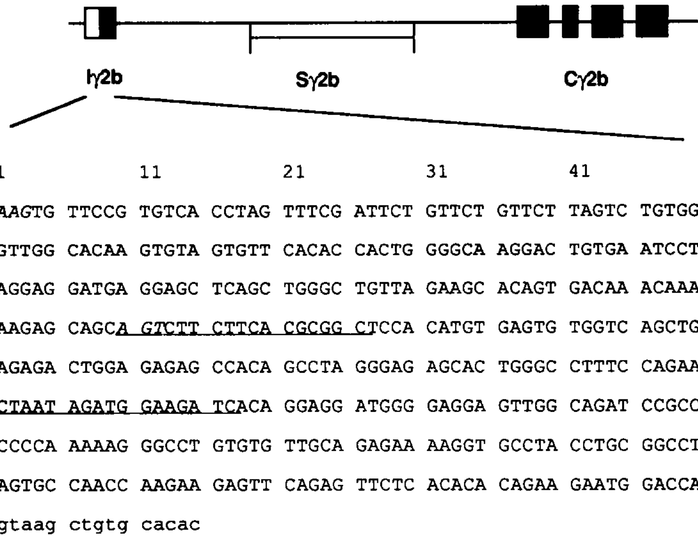
Northern Blot Analysis. Purified resting B cells were stimulated with LPS or LPS plus IL-4 in the absence or presence of S-oligos (0.3 or 0.5 μM) for 4 or 5 d. Viable cells were obtained by Ficoll-Hypaque centrifugation. Total RNA (3–10 μg) was separated by electrophoresis in formaldehyde-agarose gels and blotted onto GeneScreen membrane filters (New England Nuclear, Boston, MA). Ethidium bromide staining was used to verify that equal amounts of ribosomal RNA and presumably total RNA was loaded into individual wells. Mouse mRNAs for secretory or membrane type of Igs were detected with specific DNA fragments. A 200-bp fragment of Iγ2b cDNA was obtained by PCR by using two specific primers (sense: AGAGACTGGAGAGAGCCA and anti-sense: TGGTCCATTCTTCTGTGT). The probe was ^{32}P labeled by the random primer method to a specific activity of 4×10^8 to 1×10^9 cpm/μg. After baking, filters were prehybridized at 42°C in a solution containing 50% formamide, 1 M NaCl, 10% Dextran sulfate, and 1% SDS for >20 min. Hybridization was carried out for 12–16 h at 42°C. Filters were washed twice in 2× SSC (1× SSC is a 0.15 M NaCl, 0.015 M sodium citrate, pH 7) at room temperature for 5 min, twice with 2 × SSC-1% SDS at 60°C for 30 min, and twice with 0.1 × SSC at room temperature. Filters were exposed at −70°C onto Kodak XAR-2 film for 2 h to 2 d with intensifying screens.

Examination of Efficiency of Hybridization of Radiolabeled SE to AS and Its Variants. Both S- and O- oligonucleotides "sense" to positions 160–177 in the Iγ2b exon (SE) were radiolabeled with γ[^{32}P]ATP by using polynucleotide kinase and purified. Radiolabeled SE was incubated without or with various concentrations of AS or its variants in a buffer including 0.14 M NaCl and 10 mM Hepes (pH 7.4), heated and slowly cooled to the indicated temperature. The samples were immediately subjected to electrophoresis on a nondenaturing acrylamide gel at 4°C.

Results

To determine whether the Iγ2b/Cγ2b germline transcript expressed in B cells as a result of LPS stimulation might exert a function, we incubated such cells with a conventional antisense oligonucleotide homologous to bases 160–177 of the Iγ2b sequence illustrated in Fig. 1. Stimulation of purified B cells with LPS leads to striking induction of Ig secretion including IgG2b. Incubation of these cells with the conventional antisense oligonucleotide (0.1–33 μM) or with the homologous sense oligonucleotide had no effect on the production of IgG2b or indeed of any Ig isotype by these cells (data not shown). However, measurements of persistence and uptake of the oligonucleotide indicated that it was rapidly degraded in the culture medium and that little entered the cell intact. S-oligonucleotides have the characteristics of both being more stable and more soluble than conventional oligonucleotides (23), and so, we next employed these agents.

A Sequence of Iγ2b gene (Reference 4)



B Oligonucleotides

(1) AS oligonucleotide and its variants

1. Anti-sense to Iγ2b 160-177 (AS)	agc cgc gtg aag aag act
2. Mutated AS (MAS)	agg cgc gtg aag aac act
3. Hypermutated AS (HMAS)	agg gcg ctg aag aac act
4. Middle (167-172) mutated AS (MMAS)	agc cgc gac ttc tag act
5. 3'(172-177)-mutated AS (3'MAS)	agc cgc gtg aag ttc tga
6. 5'/3'(162-165;174-177) mutated AS (5'/3'MAS)	agg gcg gtg aag aac tga
7. 5'(160-165)-mutated AS (5'MAS)	tcg gcg gtg aag aag act
8. Anti-sense to Iγ2b 150-167 (150AS)	**aga aga ct**g ctg ctc ttt
9. Anti-sense to Iγ2b 170-187 (170AS)	tca cat gtg g**ag ccg cgt**

(2) Control oligonucleotides

10. Sense to Iγ2b 160-177 (SE)	agt ctt ctt cac gcg gct
11. Anti-sense to Iγ2b 251-268 (AS-3')	tga tct tcc atc tat tag
12. Anti-sense to Iε (ASIε)	tct tct gcc ccc tgt gca
13. Sense to Iε (SIε)	tgc aca ggg ggc aga aga

Figure 1. Sequence of Iγ2b gene and employed oligonucleotides. (*A*) Mapping of γ2b and sequence of Iγ2b gene. The Iγ2b exon (spliced onto the CH1 domain of Cγ2b) is denoted by uppercase letters. The most 5′ and 3′ transcription initiation sites reported in reference 4 are shown in italics. The sequences corresponding to the antisense oligonucleotides 160–177 and 251–268 are underlined. (*B*) Employed oligonucleotides. Antisense to Iγ2b (160–177) (*AS*) and its variants are shown in *1*. Bases changed in mutated oligonucleotides are shown in underlined italics. Bold letters show sequences overlapping with AS in the case of 150AS and 170AS. Control oligonucleotides are shown in *2*.

An Antisense Iγ2b S-Oligonucleotide Inhibits Secretion of Ig in Response to LPS or LPS + IL-4. Resting B cells were incubated with various concentrations of an antisense S-oligonucleotide homologous to positions 160–177 and with the corresponding sense S-oligonucleotide. These oligonucleotides were designated AS and SE, respectively (see Fig. 1 for abbreviations of S-oligonucleotides). AS caused a dose-dependent inhibition of secretion of IgG2b in response to LPS, but it also inhibited secretion of IgM and IgG3 to similar extents (Fig. 2). Furthermore, AS inhibited both the IgG1 and IgE responses to LPS plus IL-4. Inhibition was substantial at 0.1 μM AS (10–50-fold except for IgG1) and was somewhat greater at 1.0 μM. SE caused a modest inhibition at Ig secretion of 1.0 μM. Maximal inhibition required the addition of AS on the day of stimulation by LPS. Ig was measured in the supernatant fluid 6 d later. Substantially less inhibition was observed when the addition of AS was delayed until day two of culture, and still less if addition was delayed until day four (data not shown). Inhibition by AS could not be attributed to cell death since the yield of viable cells in response to LPS or to LPS + IL-4 was similar in these microwell cultures, at day 6, whether AS or SE was present.

AS Does Not Inhibit Expression of Membrane Ig on B Cells. Despite the striking inhibition of secretion of Ig induced by AS, no such inhibition in the frequency of cells expressing membrane IgM, IgG1, IgG2b, and IgG3 was noted (Table

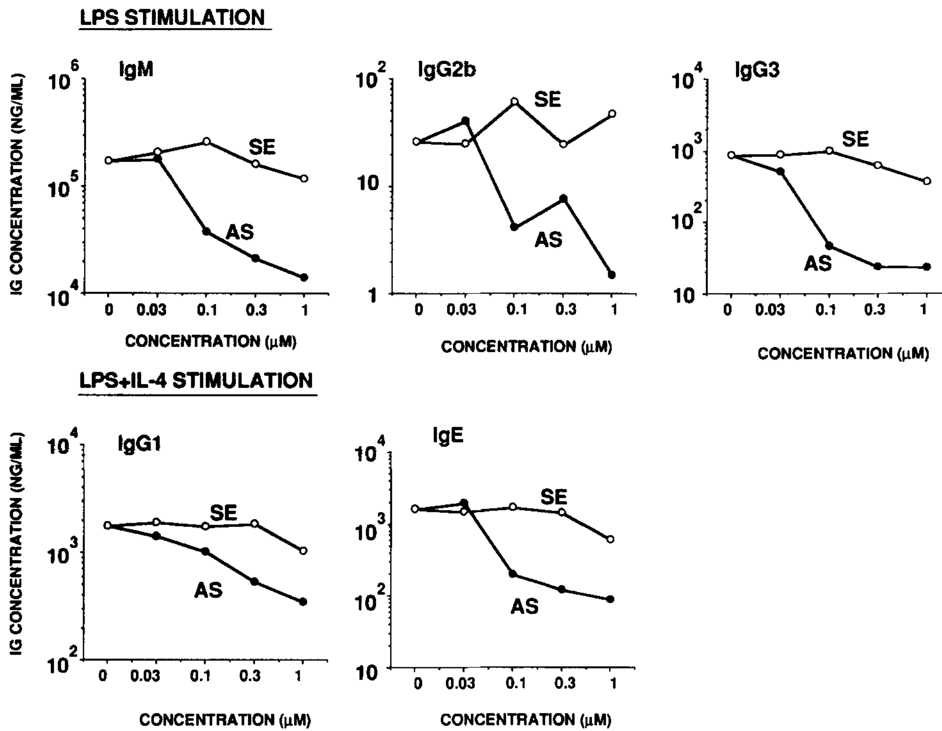


Figure 2. AS inhibits Ig secretion by stimulated B cells. Dense B cells (4×10^4 cells/well) were cultured with LPS or LPS plus IL-4 (10,000 U/ml) in the absence or presence of various concentrations of AS or SE (0.03–1 μM) for 6 d. Secreted Ig in supernatant fluid was measured by isotype-specific ELISAs.

1). This was true for B cells stimulated with LPS alone or with LPS plus IL-4. Indeed, there was a reproducible increase in the frequency of cells expressing IgG2b in both LPS and LPS plus IL-4-treated cells. There was also a modest increase in the frequency of cells expressing IgM. In addition, the mean fluorescence intensity of positive cells for the various isotypes was not diminished by treatment with AS.

AS Is Not a General Inhibitor of Protein Synthesis. The inhibition of secretion of Ig in response to LPS was not accompanied by a general inhibition of secretion of other proteins. Cells were stimulated with LPS alone or together with AS or SE for 5 d and the same number of viable cells was biosynthetically labeled with ^{35}S-methionine. TCA precipitates of both culture fluid and cell lysates were analyzed by SDS-PAGE. Fig. 3 demonstrates a striking inhibition of μ and $\gamma3$ H chains and of L chains in the supernatants of cells treated with LPS + AS compared with cells stimulated with LPS alone or LPS + SE. The identity of the indicated chains was established by

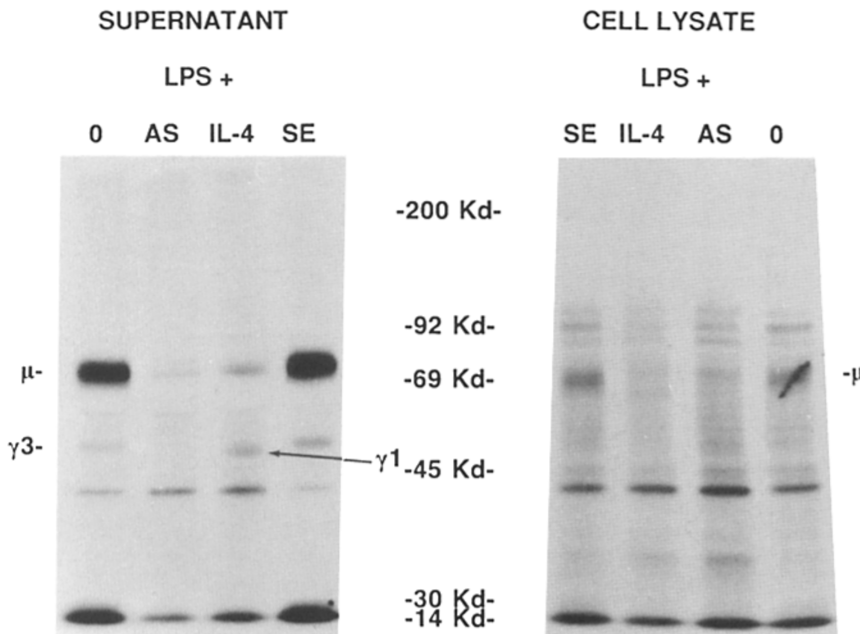
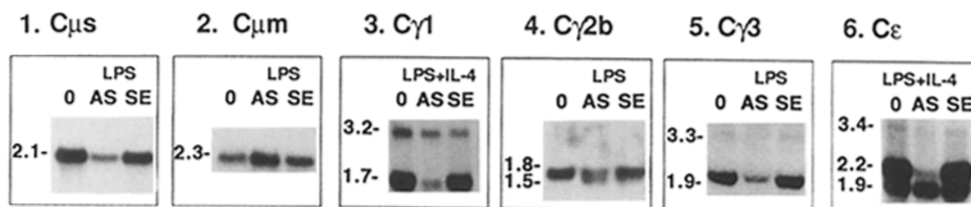


Figure 3. AS specifically inhibits synthesis anc secretion of Ig. Dense B cells (10^6 cells/ml) were cultured with LPS in the absence or presence of AS or SE (0.5 μM) or LPS plus IL-4 (10,000 U/ml) for 5 d. Viable B cells were obtained and synthetically radiolabeled with ^{35}S-methionine. After washing, B cells were further incubated for 3 h and TCA-insoluble supernatants and TCA cell lysates were obtained and dissolved in 0.5 ml of sample buffer. For the analysis of supernatants, 10 μl of each sample was subjected to electrophoresis. For cell lysis, a constant amount of radioactivity was subjected to electrophoresis. In both experiments SDS-PAGE was performed under reducing conditions. The identity of bands labeled μ, $\gamma3$, and $\gamma1$ was established by immunoprecipitation with specific antibody.



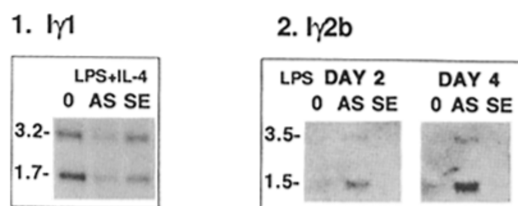


Figure 4. Expression of Ig mRNA. Dense B cells (10^6 cells/ml) were cultured with LPS or LPS plus IL-4 (10,000 U/ml) in the absence or presence of AS or SE (0.3 or 0.5 μM) for 4 or 5 d. Viable cells were obtained and total RNA was isolated from cells. 3–10 μg of total RNA was subjected to electrophoresis. The presence of equal amounts of RNA was confirmed by ethidium-bromide staining of 18 and 28 S ribosomal RNAs. After transfer, filters were hybridized with radiolabeled probes. After washing, filters were exposed 2 h to 2 d.

immunoprecipitation with specific antisera. No difference in the intensity of other protein bands was observed. In cell lysates, it was clear that AS inhibited synthesis of μ H chains, but a variety of other protein bands had similar amounts of ^{35}S. These results thus indicate that the inhibition of secretion of Ig by treatment of LPS-stimulated cells with AS was specific.

AS Inhibits mRNA for Secretory Forms of Ig H Chains and Induces γ2b Germline Transcript. RNA was prepared from viable B cells that had been stimulated for 4 or 5 d in the presence of LPS or LPS plus IL-4 alone, or with AS or SE. The presence of equal amounts of RNA was confirmed by ethidium bromide staining of 18 and 28S ribosomal RNAs before transfer to the filters in all experiments. AS caused striking inhibition of the secretory form of the productive ϵ H chain transcript in B cells stimulated with LPS + IL-4 (Fig. 4). This could be determined directly since the secretory productive transcript is 2.2 kb while the secretory form of the germline ϵ transcript is 1.9 kb. There was not detectable change in the intensity of the germline ϵ transcript. For other Ig classes, the analysis was somewhat more complex since the germline and productive transcripts have similar mobility. For γ1 and γ3, the secretory mRNA (1.7 and 1.9 kb, respectively) was substantially diminished in cells treated with AS, and the membrane mRNA (3.2 and 3.3 kb, respectively) was only modestly effected. The use of a probe specific for the secretory form of μ reveals considerable inhibition by AS while the membrane-specific μ probe reveals no inhibition (or even a slight increase) in response to AS. The result with a γ2b-specific probe was more difficult to interpret. The band at 1.8 kb, corresponding to the secretory form of the productive mRNA, appeared to be diminished in cells treated with LPS + AS, but the band was also broadened, which is consistent with an increase in the amount of the secretory form of the germline γ2b transcript, which is 1.5 kb in size.

This was clarified through the use of an Iγ2b-specific probe. This probe was prepared by PCR using primers designed to give a cDNA homologous to nucleotides 201–400 in the sequence in Fig. 1. This was chosen so as to avoid any interference by the AS or SE, which were homologous to positions 161–177. Using this probe, it was clear that treatment with LPS + AS caused a striking induction in the steady-state level of the germline γ2b transcript, both of the membrane (3.5 kb) and secretory forms (1.5 kb) at both 2 and 4 d after stimulation. Densitometric analyses indicated that the degree of enhancement of the secretory form of the γ2b germline transcript caused by AS was 10–20-fold. By contrast, analyses of germline γ1 transcripts showed a modest diminution in response to AS and an Iγ3-specific probe revealed no enhancement of the germline γ3 transcript in response to AS (data not shown). These analyses led to the striking and unexpected result that treatment with AS to Iγ2b, rather than diminishing the steady-state level of the γ2b germline transcript, actually caused a substantial increase in its level.

Table 1. *Surface Immunoglobulin-expressing B Cells*

	IgM	IgG1	IgG2b	IgG3
			%	
LPS	82 ± 2*	3.4	8 ± 2	13 ± 1
LPS + AS	93 ± 0	3.6	15 ± 3	12 ± 1
LPS + SE	85 ± 0	3.5	7 ± 0	10 ± 1
LPS + IL-4	73	22	6	3.9
LPS + IL-4 + AS	80	21	11	3.9
LPS + IL-4 + SE	76	24	6	3.3

Resting B cells were cultured with LPS or LPS plus IL-4 (10,000 U/ml) in the absence or presence of AS or SE (0.3 μM) for 5 d. After washing, cells were stained with appropriate anti–mouse Ig.

* Data are means ± SD. Experiments in which SD are shown were carried out three times. Others were performed once or twice.

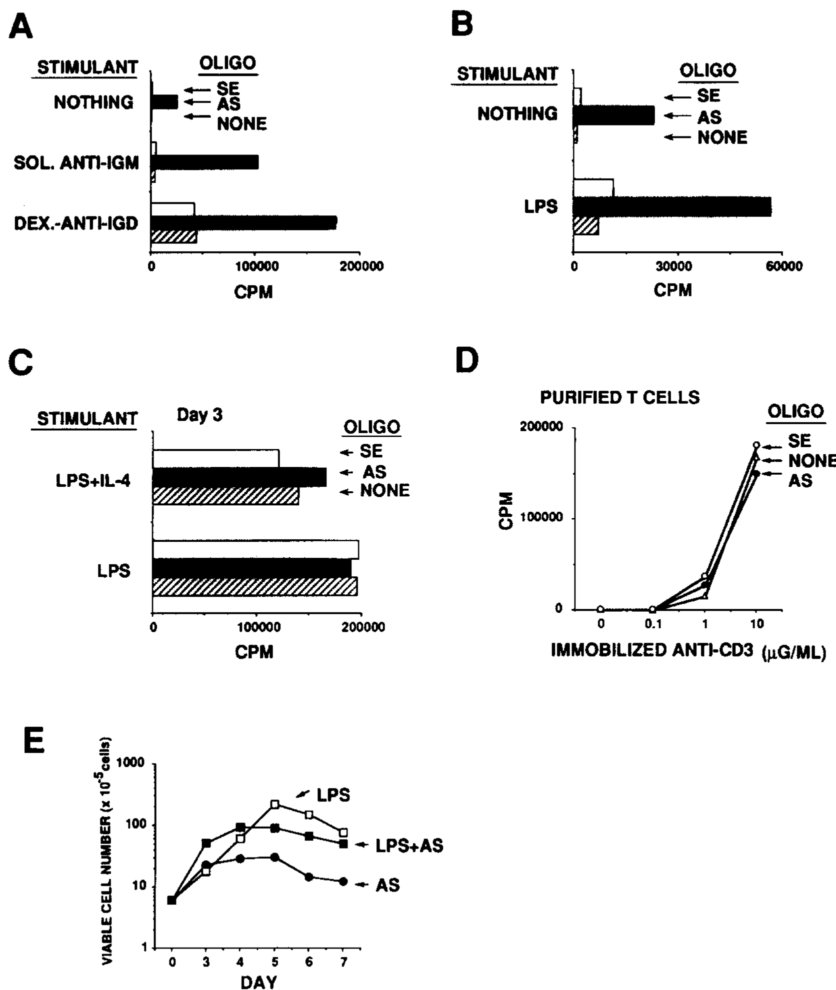


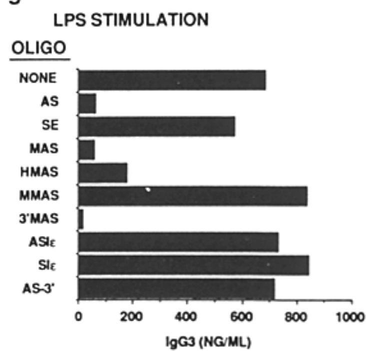
Figure 5. Induction of B cell proliferation by AS. (*A*) Dense B cells (4 × 10^4 cells/well) were cultured without or with anti-IgM antibody (50 μg/ml) or Dex-anti-IgD antibody (100 ng/ml) in the absence or presence of AS or SE (0.3 μM) for 2 d; 1 μCi of [^{3}H]thymidine was added and 4 h later incorporated [^{3}H]thymidine was measured. (*B*) Dense B cells (4 × 10^4 cells/well) were cultured without or with LPS in the absence or presence of AS or SE (0.3 μM) for 2 d and [^{3}H]thymidine incorporation was measured. (*C*) Dense B cells (4 × 10^4 cells/well) were cultured without or with LPS either alone or with IL-4 in the absence or presence of AS or SE (0.3 μM) for 3 d and [^{3}H]thymidine incorporation was measured. (*D*) Purified T cells (4 × 10^4 cells/well) were incubated in wells, coated with or without various concentrations of immobilized anti-CD3 antibody (0.1–10 μg/ml), in the absence or presence of AS or SE (0.3 μM) for 3 d, and [^{3}H]thymidine incorporation was measured. (*E*) Dense B cells (6 × 10^5 cells/plate) were cultured with AS (0.3 μM), LPS, or LPS plus AS for the indicated time, and viable cell number was counted.

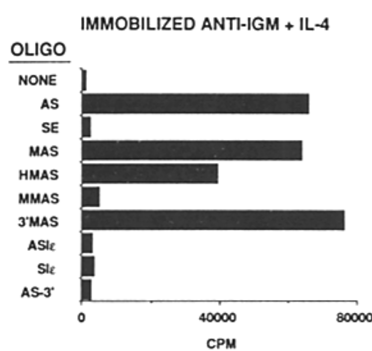
AS Induces B Cells to Synthesize DNA and Enhances DNA Synthesis in Response to Anti-Ig. Dense B cells cultured with 0.3 μM AS for 2 d displayed striking stimulation of DNA synthesis compared with the same cells cultured alone or with SE (Fig. 5 *A*). AS also caused a marked enhancement in the response of dense B cells to soluble anti-IgM antibodies or to anti-IgD–Dextran conjugates. In these cases, both AS and the anti-Igs caused responses by themselves, but the mixture caused a degree of B cell DNA synthesis that was significantly greater than the sum of the individual responses. When DNA synthesis was measured at 2 d, AS caused a significant enhancement in DNA synthesis in response to LPS (Fig. 5 *B*). However, when analyzed at 3 d of culture, AS had no effect on the response of dense B cells to LPS, with or without IL-4 (Fig. 5 *C*). SE also stimulated these B cell responses in some experiments, but their magnitude was substantially less than those caused by AS and higher concentrations of oligonucleotide were required.

AS had no effect on DNA synthesis by purified T cells in the absence of a T cell stimulant nor did it affect the DNA synthetic response of these cells to various amounts of immobilized anti-CD3 antibody (Fig. 5 *D*) although a significant amount of AS was incorporated into T cells after 2 d of incubation (data not shown). In other experiments, AS had no effect on the response of several growth factor–dependent murine cell lines, including a T cell line (CT.4S), a mast cell line (CFTL.12), and a myeloid line (FDC.1), to their respective growth factors, nor did AS cause these cells to grow in the absence of their growth factors. These results suggest that the growth-promoting effect of AS is specific for B cells.

Actual viable cell numbers were determined by trypan blue exclusion after dense B cells had been cultured with AS, LPS alone, or both (Fig. 5 *E*). AS stimulated an increase in the number of B cells in the absence or presence of LPS. The kinetic pattern of increase in cell number in the cultures stimulated with AS or AS + LPS was different from cultures stimulated with LPS only. The addition of AS caused a more rapid increase in B cell number. Taken together with the data in Fig. 5, *B* and *C* showing earlier stimulation of [^{3}H]thymidine uptake by AS or AS plus LPS than by LPS alone, these results indicate that AS caused B cell proliferation more promptly than did LPS.

Variants of AS Have All or None of Its Actions. To examine the specificity of AS and to compare its functions with its structure, we tested the effect of other S-oligos at 0.3 μM (see Fig. 1 for a list of these oligonucleotides). An S-oligo





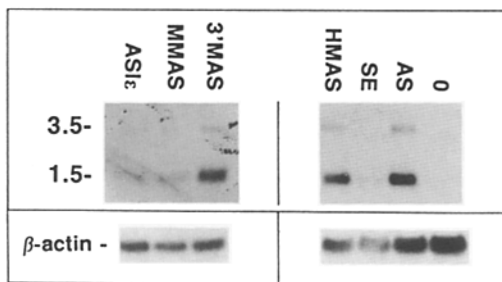


Figure 6. Effect of variants of AS. (*A*) Dense B cells (4×10^4 cells/well) were cultured with LPS in the absence or presence of oligonucleotide (0.3 μM) for 6 d and secreted IgG3 was measured by ELISA. (*B*) Dense B cells (4×10^4 cells/well) were incubated with immobilized anti-IgM antibody (10 μg/ml) plus IL-4 (1,000 U/ml) in the absence or presence of oligonucleotide (0.3 μM) for 2 d and [^{3}H]thymidine incorporation was measured. (*C*) Dense B cells (10^6 cells/ml) were cultured with LPS in the absence or presence of oligonucleotide (0.3 μM) for 5 d. After washing, total RNA was obtained from viable cells and the expression of germline transcript of Iγ2b/Cγ2b was examined. Results from two separate experiments are shown. Expression of β-actin mRNA on the same filters is also presented.

that was antisense for a more 3′ sequence in Iγ2b (AS-3′) had no effect on the production of IgG3 in response to LPS nor did it enhance DNA synthesis by B cells stimulated with immobilized anti-IgM antibody (Fig. 6, *A* and *B*). Similarly, S-oligos that were antisense or sense to a sequence in Iε (ASIε and SIε) had no effect in either assay.

S-oligos were synthesized in which the sequence of AS was varied. The resulting S-oligos differed from AS at from two to eight bases. Four, MAS (differing at two positions), HMAS (differing at six positions distributed throughout the AS sequence), 3′MAS (differing at six positions in the 3′ portion of AS), and 5′MAS (differing at six positions in the 5′ portion of AS), inhibited IgG3 production in response to LPS and caused considerable increase in B cell DNA synthesis in response to immobilized anti-IgM, although HMAS and 5′MAS were somewhat less effective than the others. By contrast, neither MMAS (differing at six positions in the middle portion of the AS sequence) nor 5′/3′MAS (differing at eight positions, four in the 5′ region and four in the 3′ region) had any activity when tested at 0.3 μM. The results for 5′MAS and 5′/3′MAS are not shown.

A test was also made of the capacity of the various S-oligos to induce the γ2b germline transcript in B cells stimulated with LPS. Fig. 6 *C* demonstrates that ASIε failed to induce expression of this transcript. AS-3′ and SIε also did not induce expression of the γ2b germline transcript (data not shown). Among the variants of AS, Fig. 6 *C* shows that 3′MAS and HMAS do induce the transcript, and MMAS does not. In other experiments (data not shown), it was shown that MAS does induce the germline γ2b transcript while 5′/3′MAS does not. Thus, among the variants of AS tested, all or none of the functions (inhibition of IgG3 production, enhancement of DNA synthesis, and induction of the Iγ2b germline transcript) was induced.

The Activity of S-Oligonucleotides Correlates with Their Capacity to Hybridize with ^{32}P-labeled SE. If the capacity of variants of AS to express activity was due to their capacity to hybridize with Iγ2b sequences, it might be anticipated that activity would correlate with the ability of the variants to hybridize with SE, since the latter represents a sequence that has been reported to be present in the Iγ2b transcript and in the noncoding strand of the Iγ2b exon (4). SE was ^{32}P-labeled and mixed with various concentrations of AS or of the AS variants. These mixtures were brought to 94°C and cooled slowly to the indicated temperature (Fig. 7, *A* and *B*). The mixtures were then subjected to electrophoresis on a nondenaturing acrylamide gel and the degree of hybridization was determined by the retardation in the mobility of ^{32}P-SE (Fig. 7 *C* presents an example). Results are reported as 2^+ if all of the ^{32}P-SE was retarded in its mobility, 1^+ if a portion had retarded mobility, and − if none of the ^{32}P-SE displayed retarded mobility.

AS hybridized most effectively with SE, showing partial retardation in SE mobility with 3.3-fold molar excess at 52°C and complete retardation at a 10-fold molar excess at this temperature. 3′MAS showed partial hybridization at a threefold molar excess at 48°C while MAS displayed partial hybridization at this temperature at a 10-fold molar excess. We failed to observe hybridization by the other variants to ^{32}P-SE at temperatures as low as 28°C. O-oligonucleotides hybridize

A Phosphorothioate ³²P-SE-oligo

Temp.	Molar Ratio	AS	MAS	HMAS	MMAS	3'MAS	5'/3'MAS	ASIε
52C	3.3	+	-	-	-	-	-	-
	10	2+	-	-	-	-	-	-
48C	3	2+	-	-	-	+	-	-
	10	2+	+	-	-	+	-	-
37C	3	2+	+	-	-	+	N.D.	-
28C	3	2+	+	-	-	+	-	-
28C	3	2+						
	10		+	-	-	+	N.D.	-
	30		2+	-	-	+	N.D.	-
28C	3							
	30		2+	-	-	+	-	-
	100		2+	-	-	2+	-	-

B Conventional ³²P-SE-oligo

Temp.	Molar Ratio	AS	MAS	HMAS	MMAS	3'MAS	5'/3'MAS	ASIε
37C	3	2+						
	30		2+	+	-	2+	-	-

C Analysis of hybridization

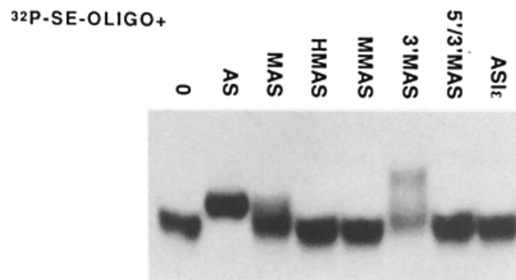


Figure 7. Hybridization of radiolabeled SE with AS variants. ³²P-labeled phosphorothioate (S)-sense or conventional (O)-sense oligonucleotide was mixed without or with various concentrations of the indicated oligonucleotide, heated, and slowly cooled to the indicated temperature. The samples were immediately subjected to electrophoresis on a nondenaturing gel. One example is shown in *C*, using a ³²P-S-SE, 28C, and a threefold-molar excess. The results of all experiments are summarized in *A* and *B*.

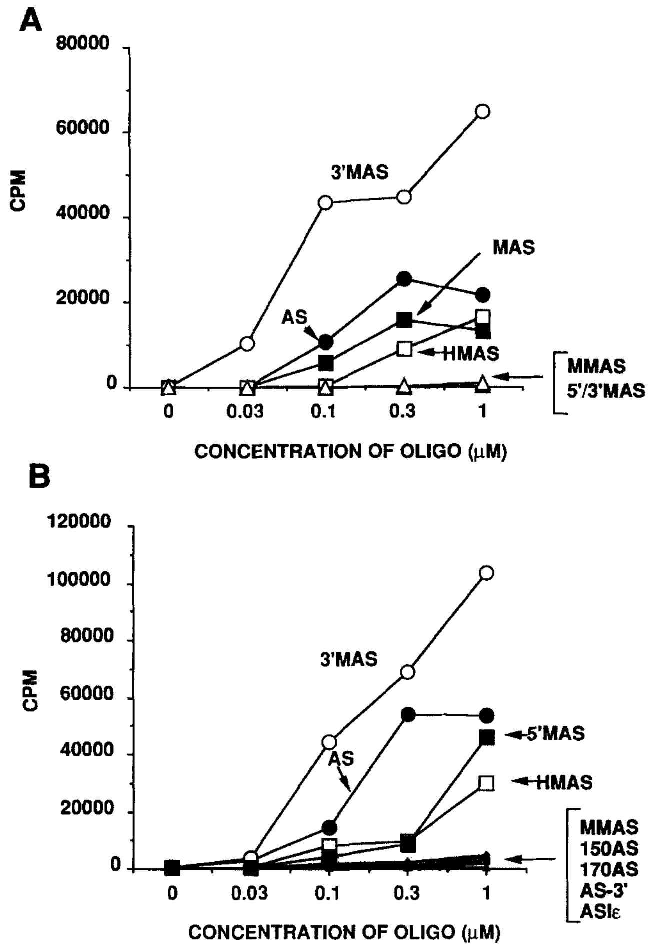


Figure 8. Comparative effects of AS variants on B cell proliferation. Dense B cells (4 × 10⁴ cells/well) were incubated with various concentrations of S-oligonucleotides (0.03–1 μM) in the presence (*A*) or absence (*B*) of immobilized anti-IgM (10 μg/ml) for 2 d and [³H]thymidine incorporation was measured.

more efficiently than S-oligonucleotides (24). Accordingly, we tested the capacity of AS and its variants to hybridize to a ³²P-O-SE at 37°C at a 30-fold molar excess, except for AS, which was tested at a threefold molar excess. AS displayed 2^+ hybridization as did MAS and 3'MAS, while HMAS displayed 1^+ hybridization. MMAS and 5'/3' MAS failed to hybridize under these conditions as did ASIε. Thus, there was a correlation between the capacity of variants of AS to express biologic activities and their capacity to hybridize to SE, although there was considerable variation in the efficiency of hybridization among the effective S-oligos. 5'MAS was not tested for its capacity to hybridize to SE.

There Is a Similar Hierarchy for Biologic Activity and Hybridization among Variants of AS. To gain more insight into the relation of the effectiveness of AS variants in biologic activity and their hybridization capacity, we carried out a quantitative comparison of the capacity of the variants to stimulate B cell DNA synthesis in the presence (Fig. 8 *A*) or absence (Fig. 8 *B*) of immobilized anti-IgM. In this assay, MMAS and 5'/3'MAS were inactive at 0.3 μM and had only limited activity at 1.0 μM. HMAS was active, but only at relatively high concentrations. MAS was more active than HMAS but less active than 3'MAS. Thus, the variants of AS displayed a hierarchy of biologic activities that was the same as their hierarchy in capacity to hybridize to SE (3'MAS>MAS>HMAS; MMAS and 5'/3'MAS ineffective). However, this correlation did not hold for AS itself, which proved to be more active than MAS but, surprisingly, less active than 3'MAS. In addition, 5'MAS was shown to be active only at high concentrations. Furthermore, two oligonucleotides that were complementary to sequences that overlapped the 5' (150–167AS) and 3'(170–187AS) ends of SE were inactive at 0.3 μM.

SE Neutralizes the Effects of AS. Although the relationship between hybridization capacity and biologic activity provides strong evidence that the action of AS and its congeners is intrinsic, the notorious capacity of B cells to respond to other stimulants such as LPS or mycoplasma products impelled us to seek more direct evidence that the action of AS was, in fact, a property of the oligonucleotide and not of

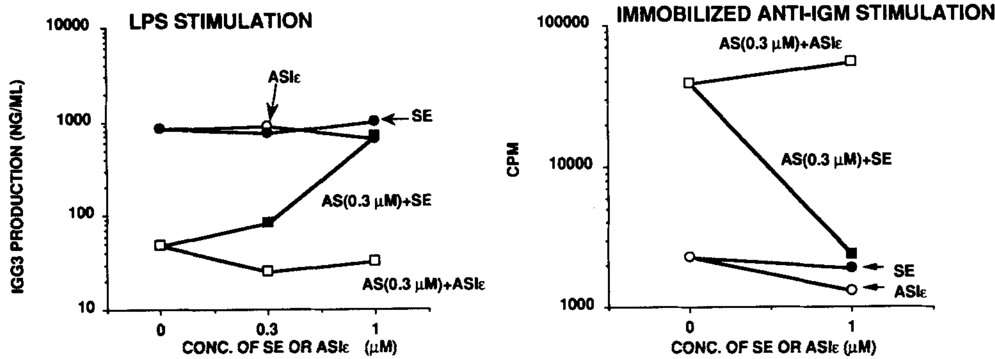


Figure 9. SE neutralizes AS-induced functions. Dense B cells (4 × 10^4 cells/well) were cultured with LPS or LPS plus AS (0.3 μM) in the absence or presence of ASIε or SE at a concentration of 0.3 or 1 μM for 6 d, and synthesized IgG3 was measured (*left*). Dense B cells (4 × 10^4 cells/well) were incubated with immobilized anti-IgM antibody (10 μg/ml) with or without AS (0.3 μM) in the absence or presence of ASIε or SE (1 μM) for 2 d and [^{3}H]thymidine incorporation was measured (*right*).

some potential contaminant in the preparation. If the activity of the AS preparation were due to a contaminant, then it would not be anticipated that its activity would be affected by hybridization to SE. By contrast, if AS were the active principle, then SE might inhibit its function. This was tested in both the inhibition of IgG3 secretion assay and the B cell DNA synthesis assay. B cells were cultured with LPS or immobilized anti-IgM in the presence or absence of AS (0.3 μM) and increasing concentration of SE. SE at 1.0 μM completely inhibited both activities of AS, but a control S-oligo, ASIε, had no effect (Fig. 8). This result also suggests that double-stranded AS/SE is not active, and thus is consistent with the conclusion that AS must bind directly to its intracellular target to cause its effects.

Discussion

The experiments described here were undertaken to examine the role of the germline γ2b transcript in Ig class switching. The premise was that antisense oligonucleotides specific for sequences in Iγ2b might either block transcription, or cause the degradation of RNA for germline γ2b. The results of these experiments revealed an unexpected and potentially very interesting property of the γ2b antisense oligonucleotide chosen for study. This S-oligonucleotide had a sequence that was antisense to that beginning at the most 3'transcription start site of Iγ2b based on a previous report (4). When incubated with LPS-stimulated B cells, it caused an enhancement rather than a diminution of the steady level of the γ2b germline transcript. This increased expression of the γ2b germline transcript was associated with inhibition of secretion of each class of Ig studied and with increased DNA synthesis by the B cells. Indeed, AS proved to be a strong B cell mitogen as judged by [^{3}H]thymidine uptake and by increase in viable cell numbers. Although the relation of the two functional effects was not investigated, it is known that B cell proliferation and Ig secretion are often inversely correlated (25) so that it is possible that the stimulation of B cell DNA synthesis may be the direct result of treatment with AS and inhibition of Ig secretion may be caused by the increased B cell entry into the S phase of the cell cycle.

Perhaps the most provocative result was the association between the increased steady-state levels of γ2b germline transcript and the biologic activities of AS. The strong correlation between these functions when variants of AS were used strongly suggests that overexpression of germline γ2b transcripts and the biologic activities of AS are functionally linked. Indeed, it seems plausible that overexpression of germline γ2b transcripts may cause the biologic effects of AS. If induction of germline γ2b transcripts plays a role in the biologic effects of AS, it would be important to determine whether other stimulants of B cell growth might exert their activity through inducing germline transcripts. LPS, which is a potent B cell mitogen, causes the expression of such germline γ2b transcripts although at a lower level than does AS.

The means through which AS increases germline γ2b transcripts have not been elucidated. The finding that each of the active variants of AS was capable of hybridizing to ^{32}P-labeled SE or a conventional oligonucleotide with the SE sequence is strong evidence that it is the capacity of the S-oligos to hybridize to the Iγ2b sequence in the germline transcript or to that sequence in the noncoding strand of the Iγ2b exon that is responsible for its effect. Initial results from experiments that are in progress indicate that the half-life of the germline γ2b transcript is not prolonged by AS, thus suggesting that AS does not exert its effect by stabilizing the RNA. These results are consistent with the possibility that AS results in increased levels of germline γ2b RNA by increasing its transcription rate. As noted above, the target for AS could either be the germline γ2b transcript itself, with the resulting double-stranded material acting to regulate transcription, possibly by competing for an inhibitory regulator that binds in the 5' portion of Iγ2b coding sequence of the gene. Alternatively, AS might act at the level of the gene by binding to the sequence represented in the noncoding strand and possibly creating a triple-stranded helix (26, 27), to which a potentially inhibitory regulator may fail to bind.

Since SE blocks the activity of AS, preformed AS/SE hybrids must be inactive. In unpublished experiments, we have observed that the cellular uptake of ^{32}P-AS/SE double-stranded oligonucleotides is similar to that of ^{32}P-AS itself. Indeed, we observed that the proportion of intracellular radioactivity that enters an NP-40-insoluble cellular compartment is similar for AS/SE hybrids and for AS, suggesting that the propor-

tion of the double- and single-stranded S-oligos that enter the nucleus is similar. Thus, if the germline γ2b transcript is the target of AS, the activity of the resulting hybrid must depend upon sequences outside of those complementary to the nucleotide itself.

The failure of ASIε to exert similar effects may mean that the Iγ2b situation is unique or it may simply mean that the incorrect sequence has been chosen to construct the Iε-complementary oligonucleotide. This is certainly a possibility since antisense–Iγ2b oligonucleotides that are complementary to 18-mer sequences that are shifted to 10 bases 5′ or 3′ compared to AS are inactive at 0.3 μM as is an antisense based on a sequence more 3′ in Iγ2b (251–268). It will be important to do a more complete survey of AS oligonucleotides homologous to sequences in other germline transcripts homologous to those in the γ2b germline transcript to determine if the action of AS is unique.

Some of the mutants are fully functional. MAS, 5′MAS, HMAS, and 3′MAS expressed the same effects as AS although at different efficiencies, whereas MMAS and 5′/3′MAS had little or no activity. These results suggest that the sequence 165–170 is essential for the full activity of AS but is not sufficient. Additional sequences either 5′or 3′ are required for biologic activities although the 5′ sequences appear more important. So far, however, sequence analysis has failed to detect any homology with any other known regulator involved in transcription, RNA processing, or stabilization.

It will be important to determine whether overexpression of germline γ2b transcripts in cells, by transfection or through transgenic technology, mimics the biologic effects of AS, and whether overexpression of other germline transcripts has comparable effects.

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Address correspondence to William E. Paul, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892.

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