Oligonucleotide inhibition of IL2Rα mRNA transcription by promoter region collinear triplex formation in lymphocytes

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Received November 26, 1990; Revised and Accepted May 17, 1991

ABSTRACT

The promoter region of the IL2Rα gene 5′ flanking sequence contains enhancer elements crucial for binding nuclear factors which upregulate transcription following T lymphocyte activation. A 3′ exonuclease resistant oligonucleotide (3′A-IL28p, terminated by a free amine group at its 3′ end) was designed to bind to the IL2Rα promoter region from −273 to −246, forming a collinear triplex spanning the κB enhancer (−266 to −256) as well as most of the serum response element (CArG box, −251 to −244). The binding site specificity of this oligonucleotide was demonstrated in electrophoretic mobility shift assays and by inhibition of restriction endonuclease (*Hin*fI) cleavage within the segment of the target DNA predicted to form a triplex with the oligonucleotide. Intact normal lymphocytes, preincubated for 2h with 3′A-IL28p, accumulated less IL2Rα mRNA relative to other mRNAs (c-myc, β-actin, IL2Rβ, IL-6) for up to 12h after PHA stimulation, than did lymphocytes treated with a control oligomer of similar composition but different sequence. Nuclear run-on studies demonstrated that the rate of IL2Rα mRNA synthesis relative to c-myc and β-actin was also selectively diminished by treatment with 3′A-IL28p. These experiments suggest that transcription of individual genes can be selectively modulated in living cells by sequence specific collinear triplex formation in regulatory enhancer sequences.

INTRODUCTION

Gene transcription is regulated by nuclear proteins that bind specific sites, often in the flanking region of the gene. The ability of these transcription factors to recognize specific sequences in double stranded DNA forms the basis of this response. Single stranded oligonucleotides can also specifically recognize double stranded DNA sequence motifs and bind to form a triple helix (1−5). Inhibitory effects of triplex forming oligonucleotides on protein-DNA interactions have been demonstrated in cell free conditions (6−9). Therefore, we hypothesized that oligonucleotides capable of forming a stable triple helix with a transcription factor binding site might block binding of the factor, selectively downregulating gene expression in living cells.

Triplex formation is based on Hoogsteen or reversed Hoogsteen H-bonding (10−11) between a duplex base pair and an oligonucleotide base, as demonstrated in structural studies of simple polynucleotides (2−5). An oligonucleotide strand of the appropriate sequence can bind to a DNA duplex in its major groove with a dissociation constant in the range of 1−10 μM or less (9,12−13). Further, triplexes can form at physiologic pH and ionic strength (7,9,14−15) suggesting that this process might permit highly specific manipulation of normal gene expression in intact cells. Since short oligonucleotides (< 50 bases) are taken up avidly by many cells (16−18), triplex-forming oligomers could have powerful applications as investigative tools, and potentially, as therapeutic agents. We now report that a triplex forming oligonucleotide can bind to a regulatory region in the 5′ flanking sequence of the IL2Rα gene and selectively suppress transcription of this gene in stimulated human peripheral blood T lymphocytes.

MATERIALS AND METHODS

Oligonucleotide preparation

Oligonucleotides were synthesized using the β-cyanoethylphosphoramidite method on a Milligen synthesizer. Subsequent to release from the solid support (Applied Biosystems (Foster City, CA) for standard nucleotide terminated oligomers or Glen Research (Sterling, VA) for 3′ amine terminated oligomers (-O-CH_2-CHOH-CH_2-NH_3+)) and deblocking, oligonucleotides were purified by C18 chromatography (trityl on), followed by detritylation, and a second round of C18 chromatography. The purity of the resulting material was determined by analytical C18 HPLC and by polyacrylamide gel electrophoresis of ^{32}P-labelled material.

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The oligonucleotide sequence was determined by placing a T for each AT base pair and a G for each GC base pair to optimize classical Hoogsteen-type pairing of the T:AT type (3,19) and the G:GC type (in the presence of Mg^{++} (20)). The more purine rich strand in the 5′ to 3′ direction was defined as parallel since oligonucleotides bind most tightly (oriented 5′ to 3′) to extended purine stretches in the major groove of duplex DNA (15,21).

Internally labelled oligonucleotides were prepared for uptake studies by the joining of half molecules with T4 ligase. A component oligonucleotide comprising the 3′ half of the molecule (A) was synthesized as either the 3′ amine or 3′ OH derivative, and then labelled under standard conditions (22) at its 5′ terminus with polynucleotide kinase and γ^{32}P-ATP. Following purification of A by electrophoresis, a 14 base oligonucleotide comprising the 5′ half of the molecule (B) was prepared. A and B were added to an 18 base oligomer (C) which had been designed as the duplex complement of the center 18 bases of the IL28p sequence, at concentration ratios A/B/C equal to 1/2/4 μM, respectively. This trinary mixture was then treated with T4 ligase at 100 U/ml for 24h at 15°C in the standard ligase reaction buffer (22). The resulting 28b product was purified from smaller molecular weight reactants by suspension in 80% formamide, heating to 80°C, and separation by polyacrylamide denaturing electrophoresis. The appropriate band was excised and electroeluted, followed by chromatography of the eluate through a Sephadex G25 exclusion column matrix to remove buffer contaminants.

Oligonucleotide inhibition of endonuclease cleavage

A synthesized duplex target sequence of 55 bp (−287 to −233) was incubated at 4 μM with or without 10 μM oligomers for 1h in 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, and 50 mM NaCl at 37°C. Ten units of *Hin*fI were then added to appropriate samples. After 10 min additional incubation, the reaction was stopped by addition of loading buffer (containing 3 mM EDTA, which also dissociates triplexes (1,20)), and the samples were electrophoresed in a 12% nondenaturing polyacrylamide gel with a buffer of 90 mM Tris/borate and 3 mM EDTA, stained with ethidium bromide overnight, and photographed with positive/negative film.

Electrophoretic mobility shift assay

DNA band shift analysis was performed as described (9). A target duplex was prepared by annealing synthesized single strands followed by electroeluting the appropriate band excised from a PAGE gel. The oligonucleotide was kinase labelled and 1000 cpm was added to each of a series of siliconized tubes. The target duplex was added to appropriate tubes in increasing concentrations, and then the buffer stock solution was added to give final concentrations of 10 mM Tris, 10 mM $MgCl_2$, 1 mM spermine, and 20% sucrose. Samples were incubated for 1.5h at 37°C and loaded directly into a native 12% acrylamide gel, with 90mM Tris/borate, 10mM $MgCl_2$ as the electrophoresis buffer. For a short duplex binding site, the mobility of the resulting triplex is slow compared to both the oligonucleotide and unbound duplex. Limited dissociation of the bound complex occurs during the course of electrophoresis (9), permitting quantitation of the labelled species by autoradiograph densitometry. The dissociation constant constant $K_d = O \times D/T$, where O, D, and T represent the concentrations of oligomer, duplex target, and triplex, respectively. Since D is known, K_d can be estimated by determining the relative proportion of O and T by densitometry.

Cells and culture conditions

Peripheral blood mononuclear cells were isolated as previously described (23) from whole blood samples donated by healthy volunteers who gave informed consent. The cells were resuspended in complete medium (23) at a concentration 10^7/ml, and 0.5 ml was pipetted into 12 ml culture tubes. An equal volume of oligonucleotide in complete medium was added to each tube at the desired final concentrations, and the cells were incubated at 37°C for 2h with gentle vortexing every 30 min to maintain them in suspension. The cultures were then stimulated with PHA (Sigma Chemicals, St. Louis) at a final concentration of 4 μg/ml. The cultures were mixed frequently during the subsequent 6−12h incubation to maintain the cells in suspension before subsequent procedures were performed.

Oligonucleotide degradation

Mononuclear cells (40×10^6) were washed and pelleted in a microfuge tube. Cells were lysed by 3 freeze/thaw cycles on dry ice, and then diluted with 3 volumes of low salt restriction enzyme buffer. Cell extract was added to a solution of oligonucleotide so that the final concentration of oligonucleotide was 1 μM and total protein was 10 mg/ml. Samples were incubated for the indicated times, before stopping the reaction by heating to 70°C for 5 min. To recover the nucleic acids, the samples were deproteinated, diluted, phenol extracted, dried by vacuum centrifugation, and desalted by soaking in 85% ethanol. The isolated material was then analyzed by electrophoresis on a 12% DNA sequencing gel after ^{32}P labelling by polynucleotide kinase (22). The resulting bands on the autoradiograph were analyzed by densitometry, and were normalized between samples by comparison of selected copurified cellular nucleic acid fragments (longer than 35 bases) present in each sample.

Cell uptake of oligonucleotides

Lymphocytes were incubated at 37°C for with trace amounts of internally labelled oligonucleotide added to 4.0 μM unlabelled oligomer. At the indicated times, two aliquots of the culture were obtained, and washed twice with PBS. One whole cell pellet was resuspended in scintillant and counted, while the other was resuspended and vortexed in 2 ml 0.5% NP-40 lysis buffer (24) to prepare isolated nuclei. After 5 min on ice, the nuclei were pelleted, treated with lysis buffer a second time, and then pelleted again. The nuclei were resuspended in scintillant and counted. Aliquots of the second lysis buffer supernatant were also tested and found to contain less than 5% of the radioactivity detected in the nuclei. In early experiments, aliquots of cells processed immediately after exposure to oligonucleotide were found to contain less than 15% of the counts present in aliquots processed at 4h. The specific activity of the culture medium was determined by counting the radioactivity in the initial supernatants for each aliquot. Mean resting whole cell and nuclear volumes were determined using a T660 Coulter Counter.

Nuclear uptake was also evaluated by recovery of oligonucleotides from aliquots of 5×10^6 intact cells exposed to 10 μM concentrations (or mock treated with no oligomer) for varying lengths of time. Cell aliquots were washed rapidly with PBS twice, and then nuclei were isolated by resuspending in 0.5% NP-40 lysis buffer. The nuclei were pelleted and the step was

repeated. The cell pellets were homogenized with a dounce tissue grinder in 1 ml buffer (80 mM Tris, pH 7.6; 5 mM EDTA; 1% SDS), and the resulting solution was extracted with phenol/chloroform. Half the aqueous phase was incubated at 60°C for 1h with proteinase K (4 mg/ml), and then extracted with phenol/chloroform. The samples were dried, soaked in 85% EtOH, resuspended in water, and 5′ end labelled with γ-^{32}P-ATP. The samples were then RNase A (10 mg/ml) treated for 15h at 37°C, reextracted, and then equal aliquots were analyzed on a 12% sequencing gel. The supernatant from the final nuclei processing step was also collected and found to contain no detectable oligonucleotide.

mRNA isolation and quantitation

Cultures (usually containing 5×10^6 cells in 1 ml) were washed and total cellular RNA was extracted using RNAzol (Cinna/BioTecx, Friendswood, TX) (25). The RNA samples were northern blotted (26), probed (27) with cDNAs of interest labelled with ^{32}P by random hexamer priming (Amersham, Arlington Heights, IL), and autoradiographed. The cDNA containing plasmids used in these studies were generously provided by W. C. Greene (IL2Rα (28)), M. Hatekeyama (IL2Rβ (29)), T. Hirano (IL-6 (30)), and L. Kedes (β-actin (31)), or obtained from the ATCC (c-myc, ATCC#41010). The quantities of each mRNA were determined by laser densitometry (LKB Ultroscan XL, Pharmacia-LKB, Gaithersburg, MD). The relative quantities of specific mRNA (IL2Rα) in cultures were compared by normalization within each sample to a control mRNA (e.g., c-myc). The difference is expressed as a percentage, calculated as shown below.

$$\%\ \text{Difference} = 100\times\frac{(\text{mRNA1/mRNA2})\ \text{in specific oligomer culture}}{(\text{mRNA1/mRNA2})\ \text{in control oligomer culture}} - 100.$$

Identification and quantitation of nascent mRNA

Mononuclear cells (5×10^6)were preincubated with oligonucleotides in triplicate, stimulated with PHA, and incubated for 10h at 37°C with frequent shaking to maintain cells in suspension. The cells were washed with ice cold PBS, and the nuclei were isolated with 0.5% NP-40 lysis buffer. Nuclear run-on assays was performed essentially as described (24), except that nuclear pellets were resuspended fresh in 100 μl transcription buffer (32) to which 100 μCi ^{32}P-UTP (3000 Ci/mM, ICN, Irvine, CA) was added. After a 30 min incubation at 30°C with frequent shaking, RNA was isolated using RNAzol (25). Filter strips bound by 2 mg of a plasmid cDNA were hybridized to the radiolabelled RNA, washed extensively, and counted directly for 15 min each in an LKB 1214 Rackbeta LSC (Pharmacia-LKB, Gaithersburg, MD). Background counts were determined using filter strips bound by 2 μg pBR322 DNA.

RESULTS AND DISCUSSION

Oligonucleotide Design

The target site selected for triplex formation in the IL2Rα 5′ flanking sequence involves several nuclear proteins important for induced transcription of this receptor gene (33–38). These factors include the NFkB family of proteins which bind to the kB enhancer (−266 to −256) and the serum response factors that bind to the CARG box (−251 to −244). The triplex-forming oligonucleotide ('IL28p') was designed to bind the IL2Rα promoter region from −273 to −246 according to our current understanding of triplex structure (see Materials and Methods). The sequence of IL28p (in parallel orientation), and that of a control oligomer relative to the target, are displayed in Figure 1A. Unmodified oligonucleotides are degraded by 3′ exonucleases present in cells (39,40). The introduction of a free amine at the 3′ terminus of IL28p, however, markedly reduced its susceptibility to degradation. Table 1 demonstrates that when unmodified IL28p was incubated with concentrated cell extracts,

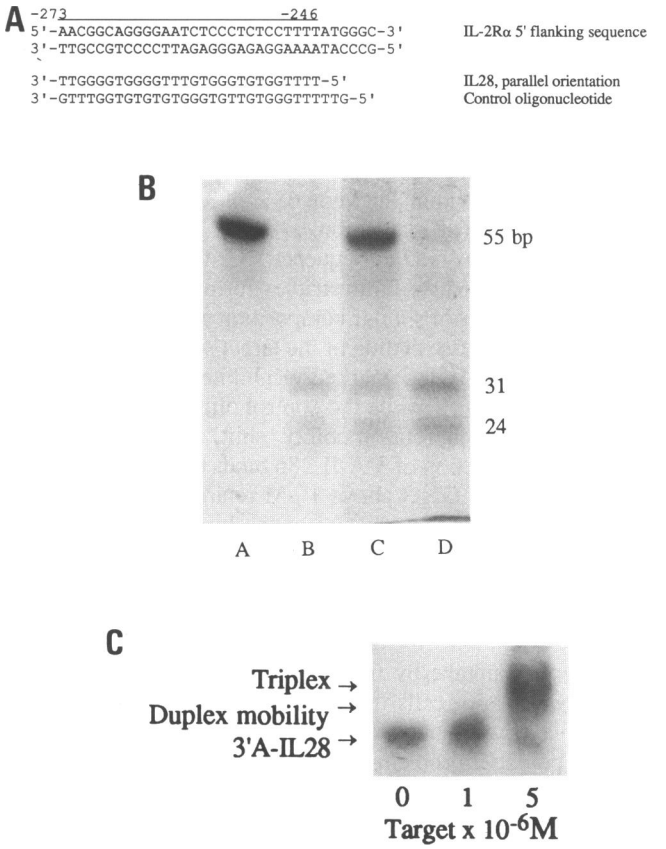

Figure 1. The sequence of the triplex forming oligonucleotide to the IL2Rα target site. (A). The sequence surrounding the −273 to −240 target sequence is presented, as well as those for the ligand IL28, an oligonucleotide which binds stably within this domain, and a control oligonucleotide, which has no detectable binding (control oligomer). (B). Inhibition of restriction endonuclease activity by triplex formation. A synthesized duplex target sequence was incubated at 4 μM without oligomer (A, B), with 10 mM 3′A-IL28p (C), or with 10 mM control oligomer (D) for 1h at 37°C. Ten units of *Hin*fI were then added to samples B, C, and D. After 10 min additional incubation, the reaction was stopped by addition of loading buffer (containing 3 mM EDTA, which also dissociates triplexes), and the samples were analyzed by electrophoresis. The resulting gel was stained with ethidium bromide overnight, destained, and photographed with positive/negative film. Densitometry of the photographic negative revealed 90% of the target remained intact in the sample treated with 3′A-IL28p (C), while 90% of the target was digested in the control oligomer sample (D). (C). The electrophoretic mobility shift assay was performed as described (9). The ^{32}P-labelled oligonucleotide and the target duplex were added in appropriate concentrations to siliconized tubes and incubated for 1.5h at 37°C. The samples were then loaded directly into a native 12% acrylamide gel and electrophoresed. For a short duplex binding site, the mobility of the resulting triplex is slow compared to the unbound duplex (which is unlabelled and thus not detected on the autoradiograph), allowing quantitation of their relative abundance. Since some dissociation of the bound complex occurs during the course of electrophoresis, a smear of radioactivity leading the triplex band is present.

Table 1. Degradation of Oligonucleotides by Cell Extracts[a]

Oligonucleotide	Time: 0h	1h	2h
IL28p	1.60[b]	0.11	0.03
% Degraded[c]		93	98
3'A-IL28p	2.60	2.02	1.76
% Degraded		22	32

[a]Mononuclear cells (40×10^6) were washed and pelleted in a microfuge tube, and extracted as described in the Materials and Methods section. Cell extract was added to a solution of oligonucleotide in each tube so that the final concentration of oligonucleotide was 1 μM and total protein was 10 mg/ml. Samples were incubated for the indicated times, before stopping the reaction by heating to 70°C for 5 min, and then the nucleic acids were recovered, kinase labelled, and size separated on a sequencing gel. The resulting bands on the autoradiograph were analyzed by densitometry, and normalized by comparison between samples of copurified cellular RNA fragments of larger size.
[b]Relative density units.
[c]Percentage loss by density comparison with the 0h band.

98% was degraded within 2h, while 68% of 3'A-IL28p remained full length after 2h of incubation.

To demonstrate the binding sequence specificity of 3'A-IL28p, we compared the capacity of this triplex forming oligonucleotide to a control oligomer of similar composition and length to inhibit restriction endonuclease cutting of the target sequence. As shown in Figure 1B, 3'A-IL28p markedly inhibited digestion of the target duplex by *Hin*fI, while the control oligomer had minimal effect. An electrophoretic mobility shift assay (Figure 1C) showed that the addition of 3'A-IL28p binds to the target duplex at concentrations of target above 1 μM retarding its mobility in the gel. The dissociation constant of the binding is less than 5 μM, since the most of the oligonucleotide is associated with the target at that concentration.

Cell Uptake of Oligonucleotides

Oligonucleotide uptake by mononuclear cells was assessed by analyzing aliquots of cells incubated for various intervals with oligomers internally labelled with ^{32}P. This labelling method avoids the potential problem of premature release of ^{32}P by cell associated phosphatase activity which could result in transfer of the label to other molecular species as well as loss of label from intact oligomer. The amount of radionuclide associated with intact cells rises rapidly and reaches a plateau at about 2h (Figure 2). The calculated oligonucleotide concentrations in intact cells and nuclei at 8h were 7 and 5.5 μM, respectively, exceeding that of the surrounding medium (4 μM). Stimulation with PHA did not alter the kinetics or magnitude of oligonucleotide uptake during the first 8h (Orson, unpublished). These findings are consistent with those reported for oligomer uptake in other studies (16, 41, 42).

To determine the condition of oligonucleotide in cell nuclei at various times during the culture period, peripheral blood mononuclear cells were incubated at 37°C with 10 μM 3'A-IL28p and aliquots of cells were removed at various time points for recovery of oligonucleotides and degradation products, followed by 5' end labelling. Polyacrylamide gel electrophoresis, as shown in Figure 3, showed that the untreated (mock) sample contained a trace of labelable nucleic acid 25 bases in length, but no copurified cellular material was detectable in the 28 base size. Scant nonspecifically adherent 3'A-IL28 was carried through in processing of samples obtained immediately after exposure to the oligonucleotide (0h). By 1h of incubation, substantial 28 and 27 base length material was demonstrable, and increasing

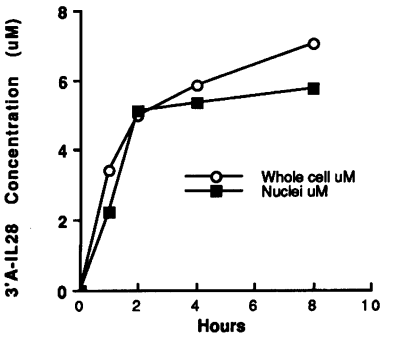

Figure 2. Cell uptake of oligonucleotides. Freshly isolated peripheral blood mononuclear cells were incubated at 37°C for the indicated times with trace amounts of internally labelled oligonucleotide added to 4.0 μM unlabelled oligomer. At the indicated times, two equal aliquots of the culture were obtained, and washed twice with PBS. One whole cell pellet was resuspended in scintillant and counted directly, while nuclei were isolated from the other by treating twice with 0.5% NP-40 lysis buffer. The second lysis buffer supernatant contained less than 5% of the radioactivity detected in the nuclei. Maximal nonspecific surface adherence in whole cell samples processed immediately on exposure to oligomer was 15% of the 4h counts in previous experiments. The specific activity of the culture medium was determined by counting the radioactivity in the initial supernatants for each aliquot which did not change over the course of the experiment. Mean resting whole cell and nuclear volumes (181 fl and 65 fl respectively) were determined using a T660 Coulter Counter.

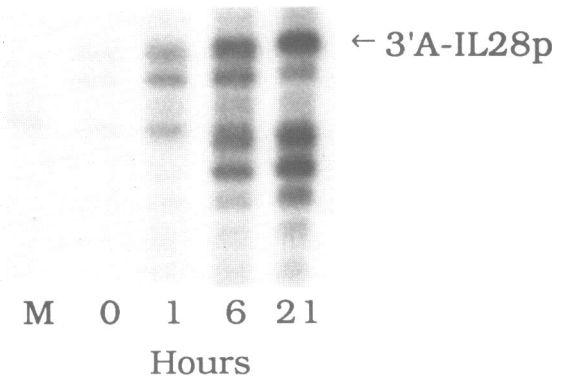

Figure 3. Recovery of full length oligonucleotide from nuclei. Peripheral blood mononuclear cells were incubated at 37°C with 10 μM 3'A-IL28p, and aliquots were removed at the indicated time points for isolation of nuclei and recovery of the unlabelled oligonucleotide. The recovered material was 5' end labelled with γ-^{32}P-ATP using polynucleotide kinase and analyzed on a 12% sequencing gel. Mock treated cells (M) not exposed to oligomer had a trace amount of cellular nucleic acid 25 bases in length, but no detectable copurified material 28 bases in length. In the aliquot obtained immediately after exposure to the oligomer (0h), scant oligomer was carried through the isolation process. At later time points (1, 6, and 21h), increasing quantities of full length material and degraded material were present.

amounts were detectable at later time points. The major portion of material recovered was full length, although increasing quantities of shorter degradation products were detectable at 6h and 21h. Taken together, these studies demonstrate that significant concentrations of oligonucleotide are taken up by the cells, with transport to the cell nucleus, and that full length oligomer persists in the nucleus throughout the culture period.

Suppression of IL2Rα Gene Transcription

To determine whether such designed oligonucleotides could specifically suppress IL2Rα mRNA responses within living cells,

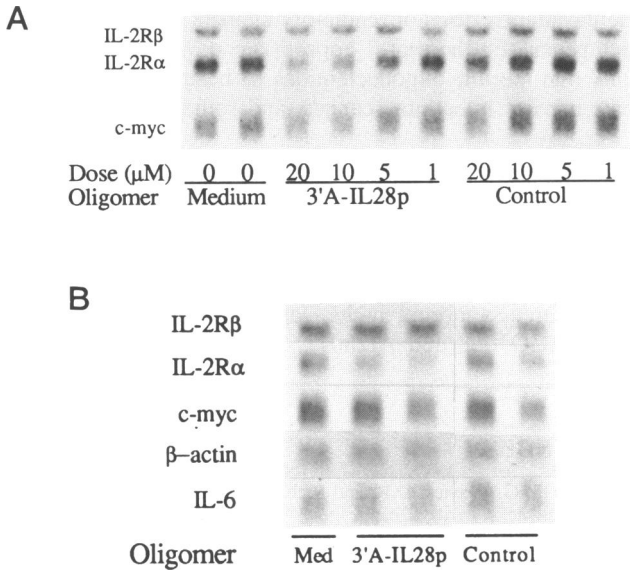

Figure 4. Suppression of IL2Rα transcription by 3'A-IL28p. (A) Autoradiograph of a northern blot of 3'A-IL28p dose response curve. After preincubation for 2h with graded doses of 3'A-IL28p and control oligomer, the cultures were stimulated with PHA and incubated with frequent mixing for 12h at 37°C. The samples were harvested, and total cellular RNA was isolated and analyzed as described in the Materials and Methods section. This autoradiograph resulted from a single hybridization of the blot with labelled IL2Rα, IL2Rβ, and c-myc cDNA. (B) Composite of autoradiographs probed with several different cDNA's. The cells were preincubated for 2h with 10 mM oligomer or medium, then stimulated with PHA and incubated with frequent mixing for 6h at 37°C. The northern blot of RNA from cells treated with either of 3'A-IL28p (duplicate cultures), control oligomer (duplicate cultures), or medium alone (single culture) was hybridized serially to the indicated cDNA's and autoradiographed.

Table 2. 3'A-IL28p Effects Normalized to Control Oligomer[a]

mRNA[b]	IL2Rα/c-myc	IL2Rα/actin	IL2Rα/IL2Rβ	IΛ2Pα/IL-6
	(10[c])	(6)	(9)	(3)
% Difference[d]	−31.1	−33.8	−26.0	−19.0
SD[e]	9.5	15.6	14.99	13.1
p value[f]	.001	.003	.001	.040
mRNA	c-myc/actin	IL2Rβ/actin	IL2Rβ/c-myc	IL-6/c-myc
	(6)	(5)	(9)	(3)
% Difference	2.3	−6.44	−.871	−4.83
SD	16.6	11.44	23.7	4.31
p value	.746	.237	.926	.192

[a]Peripheral blood mononuclear cells were preincubated with 10 μM 3'A-IL28p or 3'A-control oligomer in 1 ml medium for 2h, stimulated with optimal PHA concentrations for cell proliferation (4 μg/ml) and then incubated for a further 6h. Total cellular RNA was isolated, and the entire sample for each culture was subjected to northern blotting, ^{32}P-labelled cDNA probing, and autoradiography.
[b]Quantity of each message determined by densitometry (LKB Ultroscan XL, LKB Pharmacia-LKB, Gaithersburg, MD) of the autoradiographs.
[c]Number of experiments in which filters were probed for these messages.
[d]The differences were calculated as described in the Materials and Methods section. Comparisons of mRNA ratios were made only between samples on the same filter, i.e., transferred and probed under identical conditions.
[e]Standard deviation.
[f]P values were determined by the Student T test using 0% suppression as the null hypothesis (StatWorks, Cricket Software, Philadelphia, PA).

peripheral blood lymphocytes were preincubated for 2h with 10 μM 3'A-IL28p or control 3'A-oligomer, stimulated with PHA, and cultured for an additional 12h before extraction of total RNA. The autoradiograph shown in Figure 4A is of a northern blot

Table 3. Kinetics of 3'A-IL28p effects on IL2Rα mRNA[a]

	3h	5h	8h	12h
IL2Rα/c-myc % Difference	−23.5	−16.3	−19.7	−20.6

[a]Peripheral blood mononuclear cells were preincubated with 10 μM 3'A-IL28p or 3'A-control oligomer in 1 ml medium for 2h, stimulated with optimal PHA concentrations for cell proliferation (4 μg/ml) and then incubated for the designated times before harvesting, processing, and analyzing as described in the legend of Table II.

Table 4. Suppression of nascent mRNA transcription by 3'A-IL28p[a]

Oligomer	IL2Rα	c-myc	β-actin	IL2Rα/c-myc	IL2Rα/β-actin
3'A-IL28p	61[b]	153	80	0.40[c] (−54[d])	0.76 (−56)
3'A-Control	122	139	71	0.88	1.72

[a]Triplicate cultures of mononuclear cells (5×10^6) were preincubated for 2h with 15 μM oligonucleotide and then stimulated with PHA (4 μg/ml). After 10h incubation at 37°C, the cells were washed and nuclei prepared using 0.5% NP-40 lysis buffer. The nuclei were incubated in transcription buffer with 100 μCi ^{32}P-UTP for 30 min with frequent shaking. Nuclear RNA was isolated using RNAzol B, and the total RNA from the triplicate cultures was combined (total CPM for each condition was 22×10^6) and hybridized for 24h at 65°C to slot blot filter strips bound with 2 μg of target cDNA of interest (or pBR322 as a nonspecific binding control) in a total volume of 450 μl. The strips were washed three times, and the strips were counted for 10 min each in a liquid scintillation counter. Background was determined by the number of counts bound to pBR322 strips (average 48 CPM).
[b]Counts per min for each specific mRNA message after background counts subtracted.
[c]Ratio of specific counts for the indicated messages in the sample.
[d]Percent difference between 3'A-IL28p treated cultures and control cultures, calculated as discussed in Materials and Methods.

(probed with ^{32}P-labelled IL2Rα, IL2Rβ, and c-myc cDNA in a single hybridization) from cultures treated 12h with graded doses of 3'A-IL28p, control 3'A-oligomer, or medium alone. Due to small differences in RNA loading, densitometry of the autoradiograph was normalized to other mRNA's (c-myc and IL2Rβ) within the each sample. Compared to cultures with the equivalent concentration of control oligomer, 20, 10, and 5 μM 3'A-IL28p selectively suppressed IL2Rα mRNA by 32%, 21%, and 14%, respectively, while 1 μM had no significant effect. In contrast, there were no significant differences in the ratio of c-myc to β-actin in the samples. There was also no effect on IL2Rα mRNA splicing (43) by treatment with the oligonucleotide (Orson, unpublished); so only the 3.5 Kb IL2Rα message is shown in the figure for clarity. Of particular note is that this data demonstrates that IL2Rβ is unaffected by the IL2Rα specific oligonucleotide suggesting no regulation linkage between the two genes. This observation is consistent with the substantial sequence and regulatory protein binding site differences between the IL2Rα promoter region and the recently sequenced 5' flank of IL2Rβ (44), and is also consistent with the differences in expression of their respective mRNAs and surface proteins (29,44).

Figure 4B shows a composite of autoradiographs from a single northern blot demonstrating the relative inhibition of IL2Rα mRNA accumulation in duplicate cultures treated with 3'A-IL28p compared to control oligomer or medium alone. Densitometry demonstrated consistent selective suppression of IL2Rα relative to the various control messages in the range of 35−40%, while the largest mean difference in 4 control messages was +10%. Table 2 shows that such suppression of IL2Rα mRNA was reproducible and statistically significant in compiled results of ten experiments using 10 mM concentrations of 3'A-IL28p. The

time course of the oligonucleotide's effects on IL2Rα mRNA accumulation (Table 3) show that there is a consistent level of suppression from the earliest time point tested at 3h after stimulation through 12h.

Early in the course of IL2Rα mRNA synthesis, the accumulation of mRNA is primarily dependent on the rate of transcription (43). To demonstrate the effects of 3'A-IL28p on the IL2Rα transcription rate, we performed a nuclear run-on assay. Table 4 shows that the amount of detectable mRNA transcription at 10h for IL2Rα was decreased by 50% in terms of detectable counts in 3'A-IL28p treated cultures compared to control oligomer cultures. When normalized within each sample relative to c-myc or β-actin mRNA counts, IL2Rα was diminished by 54% and 56%, respectively. Therefore, the diminished accumulation of IL2Rα mRNA in cultures treated with the specific oligonucleotide is consistent with a decrease in the rate of transcription.

CONCLUSIONS

Triplex forming oligonucleotides capable of modulating specific gene expression are potentially useful in the investigation of gene regulation. Since they can be used in living cells, oligomers which are both effective and highly specific may also be helpful in determining the hierarchies of gene cascades activated by cell stimulation (45). Another use will be in the study of interactions between different sequences within a single regulatory region, providing an additional approach to sequence mutation and deletion in promoter-reporter gene plasmid constructs. The most immediate potential *in vitro* application may be in the modulation of abnormal or foreign genes in cells, such as those associated with malignancy or viral infection. Our laboratory has also demonstrated, for example, that an oligonucleotide designed to bind to a site in the HIV-1 LTR is able to suppress transcription of HIV-1 mRNA (McShan et. al., submitted).

The modulation of gene expression by triplex forming oligonucleotides significantly differs from the use of antisense oligonucleotides which bind to specific mRNA by Watson-Crick interactions. Antisense oligonucleotides offer a high degree of selectivity in suppressing gene expression by inhibiting mRNA splicing and translation or by increasing the rate of mRNA degradation (16, 42, 46, 47). Triplex control of transcription requires interaction with target sequences of as few as as one to two sites per cell, and avoids some potential compensatory responses to translational interference, such as alterations in native mRNA transcription rates or degradation, the latter of which is particularly important in the expression of some genes (48,49). Since 3'A-IL28p is composed of only G and T bases, Watson-Crick interactions with potential sites in either DNA or mRNA of the IL2Rα are very unlikely to have occurred in our studies. Although there are other NFκB and CArG box sites within the human genome, the length of 3'A-IL28p is sufficiently longer than the consensus sequence for either site to confer specificity for the IL2Rα gene, theoretically inhibiting crossreactivity with other sites by greatly diminishing the binding affinity for those sequences.

Transcriptional control by triplex forming oligonucleotides offers substantial potential for suppression, as shown in this paper, as well as for enhancement of gene transcription by blocking the binding of transcription inhibiting proteins, such as the factor reported for an IL2Rα negative regulatory element (50). Thus, triplex forming oligonucleotides have promise as powerful tools to delineate the effects of regulatory element interactions within intact genes. Furthermore, appropriate development of these agents may permit oligonucleotides to be used as therapeutic agents in viral infections, cancer, and some genetic diseases.

ACKNOWLEDGEMENTS

The authors would like to thank Roger D.Rossen, K.J.Hardy, J.F.Hejtmancik, and W.C.Greene for helpful criticisms and suggestions. B.T.Hall provided excellent technical support for tissue culture. Supported by the Department of Veteran Affairs, USPHS AGO7068 and AI28071, and the Department of the Navy.

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