Characterization of G_0/G_1 Switch Genes in Cultured T Lymphocytes

by

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ABSTRACT

The *G0S2*, *G0S30/EGR1*, *G0S7/FOS*, and *G0S3/FOSB* genes, identified by differential screening of human blood mononuclear cells treated for 2 hr with Con-A and cycloheximide, are thought to encode proteins that play key roles in the G_0/G_1 switch of the cell cycle. It is this switch that allows T lymphocytes to enter the cell cycle, leading to the eventual proliferation and differentiation of T cells in order to destroy foreign antigens.

To investigate the roles *G0S* gene products are thought to play, the following questions were addressed: (1) Are G0S genes responsive to Con-A independent of cycloheximide? (2) Do *G0S* gene mRNA levels increase rapidly after T cell activation? (3) Are *G0S* genes responsive to other inducing agents involved in T cell activation? (4) Can the induction of *G0S* genes be blocked by CsA? (5) Does inhibiting *G0S* gene expression by antisense oligonucleotides inhibit downstream events? A predictor of effective antisense oligonucleotides was sought by investigating a possible correlation between some aspect of RNA secondary structure and inhibitory efficacy. To gain a better understanding of factors that influence RNA structure, which in-turn, could help in designing more effective antisense oligonucleotides, the following question was also addressed: (6) Are purine-rich RNA loop domains prevalent in retroviruses and viruses committed to latency?

Using RT-PCR, *G0S2*, *G0S30/EGR1*, *G0S7/FOS* and *G0S3/FOSB* mRNA was found to increase early after T cell activation by Con-A. *G0S* gene mRNA levels were also found to increase in response to TPA and ionomycin. Early mRNA expression of *G0S2* by either Con-A or TPA+ionomycin was found to be inhibited by CsA. This was not noted for the other *G0S* genes examined.

RNA synthesis was inhibited in cultures treated with 50 nM of a *G0S30/EGR1*-complementary oligonucleotide, but inhibition decreased at higher concentrations. This decreased inhibition was also achieved using control oligonucleotides. This inhibition did not correlate with a decrease in the mRNA or protein levels of *G0S30/EGR1*.

The mRNA and protein levels of *G0S3/FOSB* were not inhibited by antisense oligonucleotides. [^{3}H]Uridine incorporation was inhibited by antisense oligonucleotides in control cultures, but not in Con-A-stimulated cultures.

A bioinformatic analysis suggested that inhibition by antisense oligonucleotides can be achieved by targeting regions of *low* base composition-determined stem-loop potential (FORS-M). However, findings of our inhibition studies targeting regions of *FOSB/G0S3* mRNA, with *high* and *low* base composition-determined stem-loop potential, contradicted our predictions. Our analysis of retroviruses (SFV-1, HIV-1, RSV) revealed that the majority of their transcripts have purine-rich RNA loops. However, viruses profoundly committed to latency (HTLV-1, EBV) have pyrimidine-rich RNA loops in the majority of their transcripts. One exception is the latency-associated transcript, EBNA-1, in EBV that is purine-rich.

The findings of this thesis support the hypothesis that G0S2, G0S30/EGR1, G0S7/FOS, and G0S3/FOSB play key roles in the G_0/G_1 switch. Our antisense oligonucleotide studies suggest that cells may possess an endogenous defense system protecting them from foreign oligonucleotides, that may be "triggered" above a critical concentration. Although unable to identify a predictor of effective antisense oligonucleotides, a phenomenon of "purine-loading" of RNA was noted in the EBNA-1 transcript of EBV, which may be useful in designing more effective antisense oligonucleotides.

CO-AUTHORSHIP

Some of the results presented in this thesis are based on a series of papers (1-4) of which there are multiple authors. The following summarizes the specific contributions made by every author to each paper.

In Chapter 2, all RT-PCR analyses of *G0S2* and *G0S30/EGR1* were carried out by me, and L. Russell carried out that of *G0S07/FOS* and *G0S03/FOSB*. RNA purification from small tissue samples was carried out by both S. P. Heximer and myself. I carried out the cloning of *G0S2* cDNA into pET19b, followed by the subsequent attempts to express and purify G0S2 protein in *E. coli*. The original manuscript was written by me, and subsequent contributions and modifications were made by D. R. Forsdyke.

In Chapter 3, all experiments were carried out by me. This includes experimental design, incorporation of radioactive nucleosides, purification of RNA and subsequent RT-PCR analyses, and preparation of cell lysates followed by immunoblotting analyses. The original manuscript was written by D. R. Forsdyke, and subsequent contributions and modifications made by me.

In Chapter 4, all experiments were carried out by me. This includes bioinformatic analyses, experimental design, incorporation of radioactive nucleosides, purification of RNA and subsequent RT-PCR analyses, and preparation of cell lysates followed by immunoblotting analyses. The original manuscript was written by me with subsequent contributions and modifications made by D. R. Forsdyke.

In Chapter 5, I carried out the Chargaff difference analysis of HVS, VZV and EBV as well as statistical significance calculations. D. R. Forsdyke carried out the analysis of

HTLV-1, SFV-1, RSV and HSV and T. P. Lillicrap carried out the analysis of HIV-1. The original manuscript was written by D. R. Forsdyke with contributions and modifications made by me.

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LIST OF ABBREVIATIONS

Chemicals

dpc	diethylpyrocarbonate
DMSO	dimethylsulfoxide
EtOH	ethanol
EDTA	ethylene diamine tetra acetic acid
PMSF	phenylmethylsulphonylfluoride
PBS	phosphate buffered saline
NaAc	sodium acetate
SDS	sodium dodecyl sulphate
Tris	Tris-(hydroxymethyl)aminomethane
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TPA	12-O-Tetradecanoyl phorbol-13-acetate

Miscellaneous

AS	antisense
BLAST	basic local alignment search tool
CD3	cluster of differentiation 3
COL	collagen
Con-A	concanavalin-A
CsA	cyclosporin A
ECL	enhanced chemiluminesence
ELAM	endothelial leukocyte adhesion molecule
FONS	folding of natural sequence
FORS-D	folding of randomized sequence difference
FORS-M	folding of randomized sequence mean
FRA	FOS-related antigen
G_0/G_1 switch gene	*G0S* gene
ICAM	intercellular adhesion molecule
IL	interleukin
MHC	major histocompatibility complex
MRP	multidrug resistance associated protein
NFAT	nuclear factor of activated T cells
NGFI	nerve growth factor inducible
P	observed significance level
PKC	protein kinase C

PAGE	Polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PTK	protein tyrosine kinase
RGS	regulator of G protein signalling
RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
SEM	standard error of the mean
SH	shuffled
TCR	T cell receptor
TdT	terminal deoxynucleotidyl transferase
TNF	tumour necrosis factor
UTR	untranslated region
VCAM	vascular cell adhesion molecule

Nucleic Acids

cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
mRNA	messenger ribonucleic acid
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid

Species

B. subtilis	*Bacillus subtilis*
D. melanogaster	*Drosophila melanogaster*
E. coli	*Escherichia coli*
S. pombe	*Schizosaccharomyces pombe*
S. cerevisae	*Saccharomyces cerevisae*
Y. lipolytica	*Yarrowia lipolytica*

Units of Measure

bp(s)	base pair (s)
cpm	counts per minute
°C	degree Celsius
Ci	Curie
D	Dalton
g	gram
hr	hour(s)

l	litre
m	metre
M	molar
min	minute
nt	nucleotide
O.D.	optical density
pI	isoelectric point
r.p.m.	revolutions per minute
s	seconds

Viruses

EBV	Epstein-Barr virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV-1	Human immunodeficiency virus type 1
HSV-1	Herpes simplex virus type 1
HTLV-1	Human T cell leukaemia virus type 1
HVS	Herpes virus saimiri
RSV	Rous Sarcoma virus
SFV-1	Simian foamy virus type 1
VZV	Varicella zoster virus

Gene and Protein Nomenclature

Italics	Gene
Non-italics	Protein

CHAPTER 1

GENERAL INTRODUCTION

Overview

In order to gain a better understanding of eukaryotic cell growth, work has focussed not only on the various stages of the cell cycle, but also on molecular mechanisms governing the regulation of cell cycle progression (5). Various aspects of these mechanisms are still not clear and therefore require further investigation. Some researchers have responded by investigating genes, the expression of which, are transiently increased at key stages of the cell cycle (6-8). Determining the kinetics of mRNA and protein expression for these genes and assessing the effects of inhibiting their expression on cell cycle progression have enabled researchers to begin evaluating their potential regulatory roles. A group of such genes, whose mRNA levels increase during the switch from G_0 to G_1 phase of the cell cycle in human blood mononuclear cells was identified by our laboratory (9). Anticipating that their gene products may serve a regulatory role in the activation of resting T lymphocytes (G_0) to enter the cell cycle and proliferate, they were termed putative G_0/G_1 switch (*G0S*) genes (Summarized in Table 1-1). The activation of T lymphocytes is a fundamental event in the entry of T cells into the cell cycle and subsequent proliferation of these cells in the launching of an immune response against a foreign antigen. In some instances, the loss of cell cycle control, such as that noted in the uncontrolled proliferation of hematopoietic cells, characteristic of myeloproliferative and leukemic diseased states, may be due to a regulatory breakdown at the level of the G_0/G_1 switch. We anticipate that this impairment could

potentially involve one or more defective *G0S* gene products. In concert with entry into the cell cycle after mitogenic stimulation. T lymphocytes are also required to launch an immediate immune response in order to destroy foreign antigens. This may be accomplished by activating the synthesis of certain gene products, such as cytokines. We suspect that some *G0S* gene products may also play key roles in this requirement. A breakdown in some aspects of this activation may lead to an impairment in the ability to launch this immune response which is characteristic of immunodeficiency and autoimmune disorders. This may involve impairment of one or more *G0S* gene products.

Mitogen-stimulated T cell activation

T lymphocytes recognize antigen fragments on the surface of antigen presenting cells (APCs) in association with the products of major histocompatibility complex (MHC) genes. CD4+ helper T cells recognize extracellular antigens associated with class II MHC gene products and CD8+ cytotoxic T lymphocytes (CTLs) identify intracellular, or endogenous antigens associated with class I MHC (10). More specifically, it is the T cell surface receptor (TCR), a heterodimer of polypeptide chains α and β, which recognizes complexes of processed antigen peptides bound to MHC molecules (11-14). The cell surface expression and function of the TCR is also dependent on the presence of several associated polypeptide chains. These include a CD3 complex, consisting of monomers of γ, δ, and ϵ polypeptide chains non-covalently associated with the TCR, as well either a $\zeta\zeta$ homodimer (90% of TCR complexes) or a $\zeta\eta$ heterodimer (10% of TCR complexes). Upon binding of antigen to TCR, the associated CD3 complex and $\zeta\zeta$ or $\zeta\eta$ chains are responsible for transducing

TABLE 1-1. **SUMMARY OF ALTERNATIVE NOMENCLATURES AND FUNCTIONS OF SOME G0S GENES**

G0S gene	Some other isolates		Role	References
	Rodent	Human		
2	*MMG0S2*	-	development?	(27)
3	*FOSB*	*FOSB*	transcription (AP1)	(6,28,29)
7	*FOS/TIS28*	*FOS*	transcription (AP1)	(30)
8	*RGS2*	*RGS2*	regulator of G-protein coupled receptor	(6,31,32, 33,34,35)
9	-	*PBEF*	cytokine	(6,36)
19-1	*MIP-1a*	*LD78α/464.1*	cytokine inhibitor of stem cells. HIV-1 suppressor	(9,37,38)
19-2	-	*LD78β/464.2*	cytokine	
19-3+CUS	-	*LD78γ/464.3*	pseudogene?	(39)
24	*TIS11/ Nup475/TTP*	-	transcription? zinc finger?	(30,40)
30	*NGFI-A/EGR1/ TIS8/Krox24/ ZIF268*	-	transcription zinc finger	(2,41)

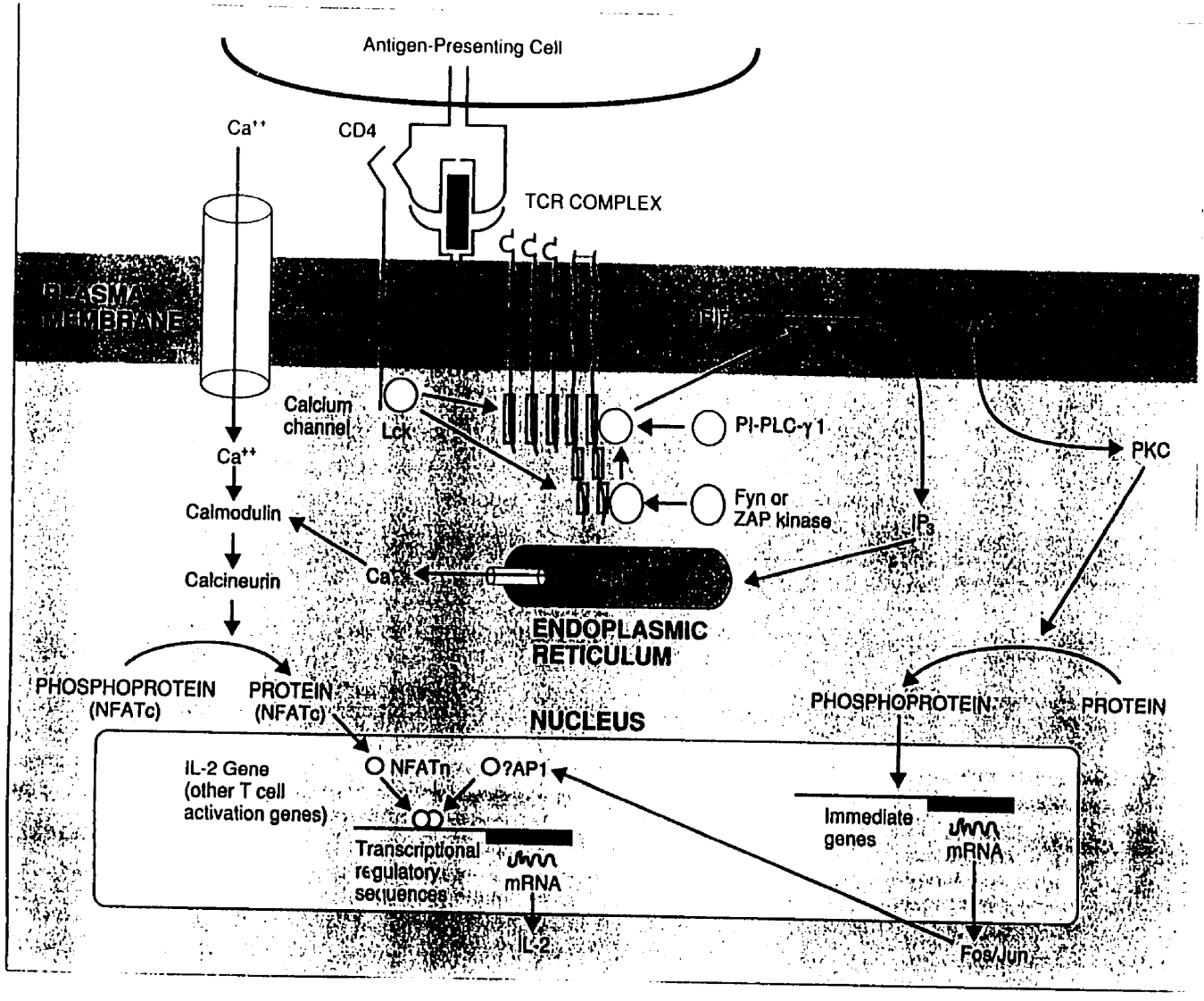
signals to the cytosol of T cells which lead to functional activation.

Upon activation, T cells in the resting G_0 phase of the cell cycle enter G_1 phase in which cell growth occurs. In this phase, the cytoplasm enlarges and there is an increase in RNA synthesis. T lymphocytes then enter S phase in which DNA division occurs and then proceed through G_2. Subsequently T cells enter the M phase of the cell cycle and undergo mitotic division. One can trace progression through the various stages of the cell cycle using radioactive nucleosides. By examining [^{3}H]uridine incorporation into RNA, one can follow progression through the G_1 phase of the cell cycle. By assaying [^{3}H]thymidine incorporation into DNA, one can follow progression through the S phase of the cycle.

Signal transduction pathways following T cell stimulation

T cell stimulation via interactions between the TCR and peptide antigen-MHC complexes on APCs results in tyrosine phosphorylation of membrane and cytosolic proteins (15-18). This includes phosphorylation of residues in the antigen recognition activation motifs (Y-X-X-L in 17 amino acid stretch) of CD3γ, δ and ϵ chains and of each ζ chain. This motif occurs once in the cytoplasmic tail of each CD3 chain and three times in that of each ζ chain (15, 17). This phosphorylation in-turn activates a cascade of intracellular signalling pathways (Fig. 1-1). Firstly, protein tyrosine kinase, ZAP-70 associates with the TCR ζ chain and phosphorylates the enzyme, phosphatidylinositol phospholipase C-γ1, resulting in its activation (17, 18). This enzyme then catalyzes the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) to inositol trisphosphate (IP_3) and diacylglycerol (DAG) (11). IP_3 increases intracellular Ca^{2+} levels, whereas DAG activates protein kinase

Figure 1-1. **Schematic of intracellular signalling pathways following activation of T lymphocyte.** This diagram is shown as illustrated in (20).


Antigen-Presenting Cell
Ca++
CD4
TCR COMPLEX
PLASMA MEMBRANE
Calcium channel
Lck
PI-PLC-γ1
PKC
Ca++
Calmodulin
Fyn or ZAP kinase
IP3
Calcineurin
Ca++
ENDOPLASMIC RETICULUM
PHOSPHOPROTEIN (NFATc)
PROTEIN (NFATc)
NUCLEUS
PHOSPHOPROTEIN
PROTEIN
IL-2 Gene (other T cell activation genes)
NFATn
?AP1
Transcriptional regulatory sequences
mRNA
Immediate genes
mRNA
IL-2
Fos/Jun

C (PKC). Ca^{2+} then interacts with the ubiquitous protein calmodulin and this Ca^{2+}/calmodulin complex activates several enzymes, including the ser/thr phosphatase, calcineurin (19). Calcineurin dephosphorylates nuclear factor of activated T cells (NFAT) so that the latter can then transport into the nucleus, bind a DNA recognition motif in the promoter region of downstream target genes which are then transactivated. Activated PKC phosphorylates various target proteins, which in-turn translocate to the nucleus and similarly transactivate downstream target genes.

T cell activation can be simulated in several ways including the use of antibodies which recognize the TCR$\alpha\beta$ heterodimer, the receptor-associated CD3 complex, or other T cell surface components such as CD2 or CD28 (21, 22). Moreover, plant lectins, such as concanavalin-A (Con-A), which recognize specific sugar residues, α-D-glucose and α-D-mannose, on T cell surface glycoproteins can be employed (23). Alternatively, one can directly activate intracellular molecules normally involved in signalling receptor occupancy. This can involve employment of either phorbol esters which mimick DAG and thereby activate PKC, or calcium ionophores which increase intracellular Ca^{2+} levels. Simulating normal T cell activation in this way has enabled researchers to identify key components of the signalling pathways that lead to the attack and removal of foreign antigens.

Studying the G_0/G_1 switch in blood mononuclear cells

Human blood mononuclear cells consist predominantly of T lymphocytes *naturally* arrested in the quiescent (G_0) phase of the cell cycle. Upon activation by Con-A, cultured lymphocytes have been found to proceed through the various phases of the cell cycle in

approximate synchrony (24). One can also study cell cycle progression in lymphocytes using cell lines rather than freshly explanted cells, however artificial arrest of these cells in G_0 is necessary. This is typically carried out by deprivation of growth factors or serum (8, 25). However, there is often a concern that such treatment causes irreversible cell damage (26), thus potentially affecting subsequent cycling of these cells. This may result in the loss of key elements involved in cellular growth control mechanisms.

Putative G_0/G_1 switch genes

It was anticipated that upon activation of T lymphocytes a group of genes would be induced, whose protein products would play key roles in the G_0 to G_1 switch of the cell cycle. It was suspected that another group of genes would be induced subsequently whose products would play key roles in G_1 progression (i.e. IL2). It is possible that the induction of these secondary genes could be dependent upon the encoded products of the primary genes (G_0/G_1 switch genes). Hence, in order to only identify genes involved in the G_0/G_1 switch and avoid detection of G_1 progression genes, cDNA libraries were differentially screened that had been prepared from human blood lymphocytes treated for two hours with both the lectin, Con-A, and protein synthesis inhibitor, cycloheximide (9). By inhibiting the peptidyl transferase activity of the 60S ribosomal subunit, cycloheximide could inhibit the synthesis of G_0/G_1 switch gene proteins and potentially avoid the induction of G_1 progression genes. By stabilizing polysomes, cycloheximide also provides a means to stabilize short-lived mRNA. Moreover, by inhibiting protein synthesis, proteins involved in a negative feedback regulation of G_0/G_1 switch genes would not be made. Using this strategy, a set of putative

G_0/G_1 switch (*G0S*) genes were identified. Noting a transient increase in *G0S* gene mRNA levels during the G_0 to G_1 switch of the cell cycle not only implicated their encoded proteins in a cell cycle transition regulatory role, but also in the activation of other key components of signalling pathways that lead to an immediate immune response. The roles of several *G0S* gene products have been determined and are summarized in Table 1-1.

G0S19 is the human homolog of murine MIP1α, a β-chemokine found to inhibit bone marrow stem cell proliferation (42). G0S19/MIP1α has been shown to bind a G protein-coupled surface receptor that is also an HIV co-receptor and thus, it has been found to show HIV-suppressive activity (38, 43). Moreover, constitutive expression of G0S19/MIP1α was noted in HTLV-I transformed T cells (44), suggesting that the virus may initiate leukemia by activating the G_0/G_1 switch.

G0S30 has been found to show high homology with its rodent homologs (EGR1, NGFI-A, TIS8, ZIF268; 30). *G0S30/EGR1* encodes a zinc-finger transcription factor that binds the DNA sequence GCGGGGGCG. G0S30/EGR1 has also been found to show homology to Wilm's tumor gene product which is a zinc-finger protein that binds this same cis-acting motif. Constitutive expression of G0S30/EGR1 has also been shown in HTLV-I- and HTLV-II-transformed cell lines (44, 45). Recently, G0S30/EGR1 has been shown to bind to its consensus sequence in the promoter region of the herpes simplex virus (HSV) latency-associated transcript (LAT) (46), *in vitro*, and thus shown to inhibit transcription. Antisense oligonucleotide inhibition studies in human myeloblastic leukemia HL-60 cells have implicated G0S30/EGR1 as a regulator of myeloid cell differentiation (47). More recent studies which examined the effects of G0S30/EGR1/NGFI-A deficiency on macrophage

differentiation and function in murine bone marrow cells derived from mice homozygous NGFI-A -/- embryonic stem cells suggest that G0S30/EGR1/NGFI-A is not required for macrophage differentiation or activation (48).

Two FOS gene family members, *G0S3* and *G0S7*, were noted among these immediate-early genes identified (6. 49). The sequence of G0S3 shows high homology with that of murine FOSB, whereas the sequence of G0S7 is identical to that of human FOS (50), a homolog of murine FOS (51). FOS protein family members (FOS, FOSB, FRA1, FRA2; reviewed in 52) have been found to heterodimerize with members of the Jun family including JUN, JUNB, and JUND, thus, constituting the AP-1 transcription factor complex. This AP-1 complex binds its DNA recognition domain in the promoter regions of various downstream genes, including that of IL-2, thus resulting in transactivation of the target gene. Mouse knockout studies of FOSB have suggested a signalling role for this transcriptional regulator in the central nervous system (CNS) (53). Very recent antisense oligonucleotide inhibition studies targeting *FOSB* mRNA in rat brain suggest that FOSB protein may play a key role in the signalling events which give rise to the dyskinetic side effects of dopamine replacement therapy for Parkinson's disease (54).

G0S8/RGS2 encodes a regulator of G-protein signalling whose expression is increased in acute leukemia (33). G0S8/RGS2 has been recently shown to bind the Gqα subunit and block Gq-directed activation of phospholipase C-β1 (55). In this way, it is suspected that G0S8/RGS2 may be involved in controlling the size of lymphoid cell populations.

G0S2 encodes a putative phosphoprotein of unknown function. Its rodent homolog,

M. musculus G0S2-like protein has been identified by differential expression in mesenchymal progenitor cells induced with human bone morphogenic protein (56).

Eukaryotic cell cycle control

Progression through the various stages of the cell cycle and check point controls is controlled by the action of both positive and negative growth regulators. The mediators of this activity include a group of enzymes known as cyclin-dependent kinases (CDKs), whose activity is regulated by subunits termed cyclins (reviewed in 26). Regulation of cell cycle progression is thought to occur at various stages of the cell cycle such as the switch from G_0 to G_1, G_1 to S and G_2 to M.

Yeast homologs of mammalian cell cycle genes

A vast amount of our current knowledge of cell cycle progression and regulation is due to years of genetic analysis of the budding yeast, *S. cerevisiae* and fission yeast, *S. pombe* (reviewed in 26). Studies have shown that yeast cells also enter a G_0-like state during (i) deprivation of nutrients, (ii) pheromone-induction of haploid mating cells and (iii) accumulation of cells at high density (reviewed in 26). Recent studies have shown a reduced expression of several TATA box-binding proteins and associated factors during this G_0 state implicating them in key regulatory roles of cell cycle progression (57). Continued studies investigating cell cycle regulation in yeast reveal a number of parallels between regulatory genes identified in yeast and those of higher eukaryotes. Such a similarity is noted in the G_1 arrest induced by pheromone in *S. cerevisiae* and that of G0S19/MIP1α (58-60). Not only

has G0S19/MIP1α been found to negatively regulate T cell proliferation, but its receptor on the surface of T cells, like that of pheromones, is a member of the G protein-coupled receptor superfamily (61, 62). In budding yeast, the homolog of G0S30, Mig1, has been found to respond to nutritional cues. It mediates glucose repression of genes, whose expression increase on starvation (63). G0S24 has been found to show homology with the *S. pombe* Mei2 protein (40), whose dephosphorylation is necessary for starvation-induced cell cycle arrest and entry into meiosis. G0S8/RGS2 has been found to functionally replace SST2 in yeast in desensitizing pheromone receptors, thus permitting cell cycle progression (32, 64, 65).

Inhibition studies

In order to investigate whether putative *G0S* gene products play important roles in cell cycle progression one would like to inhibit T cell activation and determine if the expression of any *G0S* genes are affected. One can achieve this inhibition using the general inhibitor of T cell activation, CsA. Conversely, one could use antisense oligonucleotides to inhibit the expression of specific *G0S* genes and determine whether cell cycle progression is affected.

Since its original purification from the fungus *Tolypocladium inflatum*, the immunosuppressant CsA has revolutionized transplantation medicine (66). After interacting with the ubiquitous cytosolic protein, cyclophilin A, the CsA-cyclophilin A complex is able to inhibit the activation of Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin. As a result of this inhibition, normal dephosphorylation of NFAT by calcineurin is blocked. This

cytosolic NFAT cannot transport to the nucleus and therefore cannot bind and transactivate downstream target genes (67). As an inhibitor of T cell activation, CsA is not only a powerful therapeutic agent in preventing graft rejection following organ and bone marrow transplantation, it is also of biological importance in helping to identify target genes suspected of playing key roles in T cell activation. From studies of T cell activation using CsA, two schools have emerged. One group of researchers have reported that T cell activation can be re-established by recombinant IL2 addition to CsA-treated T cells thus suggesting that IL2 is both necessary and sufficient for the immunosuppressive action of CsA (68, 69). Others have reported that recombinant IL2 addition cannot re-establish T cell activation and have thus suggested that other more immediate-early genes exist, whose expression may be CsA-sensitive (70-72). This raises the possibility that some of our putative *G0S* genes may belong to this group of immediate-early genes.

A plethora of studies have exploited the ability of antisense oligonucleotides to inhibit gene expression (reviewed in 73, 74), and there are a variety of mechanisms by which this inhibition is thought to occur. The most commonly accepted mechanism of action is the binding of oligonucleotides to target gene mRNA, thus forming a DNA/RNA heteroduplex. This heteroduplex then activates RNase H which in-turn degrades the single stranded RNA that is complexed with the DNA. As a result the protein product cannot be made. Another proposed mechanism is that oligonucleotides bind target gene mRNA and structurally interfere with the binding of ribosomes and/or the translational machinery. As a result translation is blocked. There are also other proposed mechanisms by which antisense oligonucleotides are thought to inhibit target gene expression at the level of trancription

rather than translation. Antisense oligonucleotides are able to bind DNA, thus forming a triplex and halting transcription. Oligonucleotides can bind unspliced nuclear mRNA and interfere with splicing and/or bind spliced nuclear mRNA and block transport from the nucleus to cytoplasm.

The success of inhibiting gene expression using antisense oligonucleotides has heightened interest in their potential to function as anticancer and antiviral agents. In assessing this therapeutic potential, conditions for the optimization of target gene inhibition have been explored. To this end, much research has been dedicated to enhancing the uptake and delivery of oligonucleotides as well as increasing their nuclease-resistance by modification of the phosphodiester backbone (75-78). In recent years there have also been many studies to determine predictors that would help to design more effective antisense oligonucleotides. At present, the factors which determine the effectiveness of antisense oligonucleotides are not clearly understood. Although studies have searched for a general predictor of effective antisense oligonucleotides, results have proved inconclusive (79-83).

The employment of antisense oligonucleotides has not been without technical difficulties. In fact, many have reported numerous non-antisense effects which have both intrigued and confused researchers. Such inhibitory affects have been noted when using oligonucleotides that possess 5'-PuPuCpGPyPy-3' motifs or G-quartets (reviewed in 84). Oligonucleotides have also been reported to inhibit cellular proliferation by a mechanism which is dependent on the phosphorothioate modification of oligonucleotides and not a sequence specific inhibition of target mRNA. Such findings emphasize our limited knowledge of the actions of antisense oligonucleotide and thus, identify a clear need to

further investigate these potentially powerful biological and therapeutic agents.

Specific Objectives

When the work contained in thesis was begun in January of 1994 it was postulated that *G0S* genes encode protein products that play key roles in the G_0/G_1 switch. However, evidence to support this was lacking. Therefore, to investigate these hypothesized roles in this cell cycle switch the following questions were addressed:

(1) Are *G0S* genes responsive to Con-A independent of cycloheximide?

The differential screening procedure that identified these *G0S* genes involved the use of both a T cell polyclonal mitogen, Con-A and protein synthesis inhibitor, cycloheximide. If *G0S* gene products play a key role in this cell cycle switch, we would anticipate that they would be responsive to Con-A alone and not merely to cycloheximide.

(2) Do *G0S* gene mRNA levels increase rapidly after T cell activation?

We would anticipate that if *G0S* gene products play an integral role in the G_0/G_1 switch, then they would be expressed very rapidly after T cell activation, probably peaking within the first 2 hrs and then declining.

(3) Are *G0S* genes responsive to other inducing agents involved in T cell activation?

We suspect that if *G0S* genes encode key regulatory components of the G_0/G_1 switch, then agents (i.e. phorbol esters and calcium ionophores) that can by-pass stimulation of T cells via the TCR and directly trigger intracellular molecules normally involved in signalling receptor occupancy should induce their expression.

(4) Can the induction of *G0S* genes be blocked by CsA?

If *G0S* gene products are critical to this cell cycle switch, then inhibiting T cell activation with the immunosuppressant, CsA, could inhibit their mRNA expression. It would also be necessary to demonstrate this inhibition at the protein level and show that this block in T cell activation can be reversed by introducing exogenous G0S protein. To this end, the expression of recombinant G0S2 protein was undertaken.

(5) Does inhibiting *G0S* gene expression by antisense oligonucleotides inhibit downstream events?

If *G0S* gene products play key roles in the switch from G_0 to G_1, then inhibiting their expression using antisense oligonucleotides should result in the inhibition of downstream events. To identify mRNA sites to which antisense oligonucleotides should be targeted for maximal inhibition, a bioinformatic analysis was also undertaken examining potential correlations between RNA secondary structure and antisense oligonucleotide inhibitory efficacy.

(6) Are purine-rich RNA loop domains prevalent in retroviruses and viruses committed to latency?

The inability to find a consistent predictor of effective antisense oligonucleotides suggests that a more fundamental understanding of factors that influence RNA structure is required. To this end, a Chargaff difference analysis of several viral genomes was carried out to assess the presence of purine-rich RNA loops.

Overview of Chapters

Chapter 2 investigates the induction of *G0S2*, *G0S30/EGR1*, *G0S07/FOS* and *G0S03/FOSB* mRNA in response to Con-A, cycloheximide, TPA and ionomycin. It also assesses the effect of CsA on *G0S* gene mRNA induction.

Having established the induction profiles of *G0S* gene mRNAs, Chapter 3 describes the use of a *G0S30*-antisense oligonucleotide to inhibit its expression. This would enable us to examine what effect this inhibition would have on downstream events. This chapter also describes studies that test the hypothesis that cells have an endogenous defense system protecting them from foreign oligonucleotides.

A shortcoming in using antisense oligonucleotides to inhibit gene expression is that the choice of an mRNA target site is arbitrary. Chapter 4 begins with a bioinformatic analysis to assess a potential correlation between some aspect of RNA secondary structure and inhibitory efficacy. This chapter also tests two *G0S03/FOSB*-antisense oligonucleotides (designed using results from our computational analysis) to inhibit *G0S03/FOSB* expression. This would enable us to examine what effect this inhibition has on downstream events.

Our inability to find a consistent predictor of the effectiveness of antisense oligonucleotides in Chapter 4 suggests that our understanding of RNA structure is incomplete. Chapter 5 therefore describes a Chargaff difference analysis of retroviral and herpes viruses in order to further our understanding of this structure. Such analyses could identify factors that influence RNA structure that may be helpful in designing more effective antisense oligonucleotides. This chapter identifies a previously unrecognized phenomenon of "purine loading" of mRNA which could be useful in this respect.

CHAPTER 2

Cyclosporin A Inhibits Early mRNA Expression of G_0/G_1 Switch Gene 2 (*G0S2*) in Cultured Human Blood Mononuclear Cells.

ABSTRACT

Cyclosporin A (CsA) may achieve its immunosuppressive effects by inhibiting the calcium and calmodulin-dependent protein phosphatase calcineurin, which is required for activation of target genes by members of the NFAT (nuclear factor of activated T cells) transcription factor family. Among these target genes is one which encodes interleukin-2 (IL2), a cytokine facilitating progression through the G_1 phase of the cell cycle. However, recombinant IL2 does not reverse CsA inhibition, suggesting that at least one other NFAT-sensitive gene may be involved. The human G_0/G_1 switch gene, *G0S2*, has potential NFAT binding sites in the 5' flank, and encodes a small basic putative phosphoprotein of unknown function. Using a sensitive, reverse transcription-polymerase chain reaction (RT-PCR) assay, *G0S2* mRNA levels were assayed in cultured blood mononuclear cells under various conditions. Freshly isolated cells contain high levels of *G0S2* mRNA which rapidly decline. This "spontaneous stimulation" is also noted with some other *G0S* genes, and has been attributed to some aspect of the isolation procedure. In cells which have been preincubated for 24 hr to lower mRNA levels, there is a transient increase in *G0S2* mRNA, peaking between 1-2 hr, in response to Concanavalin-A (Con-A), or to the combination of the phorbol ester 12-O-Tetradecanoyl phorbol-13-acetate (TPA) and the calcium ionophore, ionomycin. Both of these responses are inhibited by CsA. Our results suggest that *G0S2*

expression is required to commit cells to enter the G_1 phase of the cell cycle. Moreover, while not excluding other possible targets, early inhibition of *G0S2* expression by CsA may be important in achieving immunosuppression. Therefore, the immediate-early gene, *G0S2*, whose expression peaks relatively sooner than that of *IL2*, may be of value as a reporter gene for analyzing the mechanism of action of CsA.

INTRODUCTION

T lymphocytes remain in the "resting" G_0 phase of the cell cycle until activated by interactions between surface receptors and specific peptide-antigen MHC protein complexes on other cells (85). Some aspects of this signalling may be simulated by adding lectins, such as Con-A, to cultured T cells (23, 86). After an interaction period of less than 2 hr, during which time intracellular calcium levels are increased, cells are committed to enter the G_1 phase of the cell cycle (87-89). The switch to the G_1 phase is associated with increased expression of various genes, including a gene encoding a regulator of trimeric G protein-coupled signalling (G0S8/RGS2; 31, 35).

Recovery from pheromone-imposed cell cycle arrest ("G_0") in yeast is facilitated by the yeast homolog of G0S8/RGS2 (90), and this switch (i.e. reentry into the G_1 phase of the cell cycle) is inhibited by CsA (91). Similarly, CsA inhibits the G_0/G_1 switch in mammalian T cells (92-95), which may account for its profound immunosuppressive effects (96). In both yeast and mammals CsA inhibits the calcium and calmodulin-dependent protein phosphatase, calcineurin (97, 98). In T cells, calcineurin targets members of the transcription factor NFAT family (99). When dephosphorylated, cytoplasmic NFAT is able to translocate to the nucleus

where it affects the transcription of various genes including that which encodes IL2, a secreted cytokine required for G_1 progression, but not for commitment to enter the G_1 phase from the G_0 phase (100, 101).

G0S2 is one of a set of putative G_0/G_1 switch (*G0S*) genes, which were identified by differential cDNA hybridization in Con-A-activated human blood mononuclear cells (6, 9). It encodes a small basic putative phosphoprotein (103 amino acids) of unknown function. Potential target sites for transcription factors AP1, AP2 and AP3 are present in the genomic sequence (27). There are also potential target sites for NFAT, suggesting that *G0S2* expression may be regulated by NFAT, and therefore inhibited by CsA.

We here present a sensitive RT-PCR (reverse transcription-polymerase chain reaction) analysis of the kinetics of expression of *G0S2* mRNA in response to various stimuli. We demonstrate inhibition by CsA under conditions in which the expression of similar "immediate-early" genes, such as *G0S7/FOS* (component of the AP1 transcription factor complex) and *G0S30/EGR1* is not inhibited. We also demonstrate some inhibition by CsA in the expression of another component of the AP1 transcriptional complex, *G0S3/FOSB*, yet to a lesser extent than that shown for *G0S2*. Although a role of the G0S2 protein is unknown, our results suggest that early inhibition of the G_0/G_1 switch by inhibition of *G0S2* expression might be important in achieving immunosuppression by CsA. Conversely, the fact of inhibition by CsA would suggest a key role of the G0S2 protein in lymphocyte activation. As such, *G0S2* may serve as a powerful reporter gene for the further analysis of the mechanism of action of CsA and its reported roles in inhibiting both the positive and negative selection of lymphocytes (102, 103). This initial characterization of

G0S2 not only sheds some light on the pathways that influence *G0S2* expression, but is fundamental to gaining a better understanding of the pivotal role it may play in T cell activation.

MATERIALS AND METHODS

Cell Culture

Methods were as described previously (1-3, 6, 28, 35, 40). Freshly collected heparin-treated blood from healthy human donors (110 ml) was diluted with an equal volume of pre-warmed (37 °C) RPMI 1640 medium (Life Technologies, Gaithersburg), layered over 16 ml of Ficoll-Paque (Pharmacia, Piscataway, NJ) and centrifuged for 12 min at 800 x g. The mononuclear cell layer was then diluted in five volumes of RPMI 1640 and cells were pelleted by spinning for 4 min at 600 x g. Cells were resuspended in incubation medium (RPMI 1640, 83.3 %; autologous serum, 16.7 %; 2-mercaptoethanol, 0.05 %) to a final concentration of 0.5 - 5.0 x 10^6 cells/ml. Unless otherwise indicated, cells were aliquoted and incubated for 24 hr prior to addition of reagents to appropriate tubes (time defined as 0 hr). All experiments were repeated at least three times with cells from different human donors. TPA (Sigma Chem Co., Mississauga) and ionomycin (Calbiochem, San Diego) were dissolved in DMSO. TPA was added to a final concentration of 100 nM whereas ionomycin was added to 2 μM. Cyclosporin A (Sandoz, Dorval) and cycloheximide (Sigma Chem Co., Mississauga) was dissolved in ethanol. Cyclosporin A was added to a final concentration of 100 nM whereas cycloheximide was added to 100 μM. Con-A (Calbiochem, San Diego) was dissolved in water and added to a final concentration of 200 μg/mL. These solvents were

added to control cultures as required.

Preparation of total RNA

In the preparation of RNA, the use of Trizol reagent (Gibco/BRL, Life Technologies) proved to be the most reproducible of all methods attempted in our laboratory. Trizol is a commercial preparation based on the acid phenol/guanidinium thiocyanate method initially described by Chomoczynski and Sacchi 1987 (104). At various times, 2.5 ml of cells were harvested by centrifugation and pellets were shaken vigorously for 5 min in 1 ml of Trizol reagent. After addition of 0.2 ml of $CHCl_3$, samples were shaken again for 30 sec and then centrifuged to separate the upper aqueous layer containing RNA. The latter was precipitated with isopropanol and digested with RNase free DNaseI, which was then removed by phenol/$CHCl_3$ extraction followed by ethanol precipitation. Purified RNA samples were resuspended in 400 μl dpc-treated water and quantitated by spectrophotometry. Protein contamination was assessed using A_{260}/A_{280} ratio. Aliquots of the quantitated material (usually corresponding to 125-250 ng RNA) were added directly to the reverse transcription reactions. RNA was stored at -70°C.

Reverse transcription reaction

Total RNA was reverse transcribed using *G0S* gene-specific primers (17 bp) rather than random oligonucleotides (*G0S2*, 5'-AATGCAAAATGGTGGTC-3'; *G0S30/EGR1*, 5'-GCCAAACAGTCACTTTG-3';*G0S7/FOS*,5'-GGCCTGGCTCAACATGC-3';*G0S3/FOSB*, 5'-CACAAGTACAGCATGGG-3'). This served to increase both the sensitivity and

specificity of the reaction. Specificity was also increased by carrying out the reaction at 42°C. At temperatures lower than 42°C, multiple banding patterns were found when certain reverse transcription primers were used. Reverse transcription primers were chosen on the basis of proximity to (within 5-151 nucleotides of) the downstream primer of the PCR pair. This served the purpose of increasing the probability of extending cDNA synthesis completely through the desired amplicon. In order to be consistent and avoid possible variation in reaction efficiencies arising from different primer combinations, the same group of primers were used to assay RNA samples (*G0S2* and *G0S30*; *G0S3* and *G0S7*). Completed reverse transcription reactions were heated for 10 min at 94°C to remove any remaining reverse transcriptase activity, known to inhibit PCR efficiency (105). Reverse transcription mixtures were diluted in TE8 buffer (10 mM Tris-Cl, pH 8, 1 mM EDTA) in order to dilute the concentration of reverse transcription primer prior to PCR.

RT-PCR cDNA control templates

Competitor controls were prepared as previously described (1, 2, 28, 35). These competitor controls were cDNAs in plasmid pBR322, with each cDNA containing a small exogenous DNA insert (150 nucleotides) in the region to be amplified; this slightly decreased the mobility of the PCR product. Two-fold dilutions in TE8 were made of control plasmids with the appropriate cDNA insert and stored at 4°C prior to use. This procedure was routinely carried out to avoid excessive freeze-thawing of control samples which was found to decrease the amplicon signal. Our control stock samples were stored at -20 °C and thawed prior to use.

Polymerase chain reaction profile with a fixed concentration of competitor

Equal aliquots from each reverse transcription reaction mixture (containing cDNA corresponding to 5.2-10.4 ng of total RNA) were combined with a fixed number of molecules of cDNA controls (see below), and were then coamplified and the products identified by agarose gel electrophoresis with ethidium bromide. This generated a kinetic profile for the experiment ("profile assay"), which is displayed in some figures. Primer pairs for PCR in separate reactions were : AGAAGCAAGCCCTGCAGGAGAAAG (upstream), and CATGACAATGCAGTGCTGCACAGC (downstream) for *G0S2*; GTCTTGGTGCCTTTTGTGTGATGC (upstream), and GCCGCCTACTCAGTAGGTAACTAC (downstream) for *G0S30/EGR1*; AATGAGCCTTCCTCTGACTCGCTC (upstream), and GCTACATCTCCGGAAGAGGTAAGG (downstream) for *G0S7/FOS*; CCATCCTGATCCCAGACCCATCC (upstream), and ACGAACCCAGAGTCAGACGACAGC (downstream) for *G0S3/FOSB*.

Polymerase chain reaction with varying competitor concentration

Based on the intensity of ethidium bromide staining in the above profile assay, a range of two-fold concentrations of control cDNA plasmid was selected for each cDNA sample, and the PCR was repeated to quantitate more precisely the level of cDNA (106). In calculating mRNA concentrations, it must be noted that cDNA molecules begin as single strands, whereas controls begin as duplexes. Furthermore, since we were concerned with *relative* changes in mRNA levels, rather than with *absolute* quantities, there were no controls

for the efficiency of reverse transcription (107). It was assumed that, for a given gene, this efficiency would vary randomly and to approximately the same extent with different RNA samples. This argument also applies to any vagaries of the cDNA control template (i.e. there might be a stem-loop in cDNA insert which might impede PCR amplification efficiency). Our lab has carried out RT-PCR for one of the *G0S* genes, *G0S24*, and shown that whether employing control RNA or control DNA templates the value for molecules/μg total RNA obtained is comparable (108). This finding supports our assumption of comparable reverse transcription efficiencies. RT-PCR was routinely carried out with DNA controls rather than RNA controls because RNA controls are more technically demanding (i.e. easily degraded by nucleases), and are thus more error-prone..

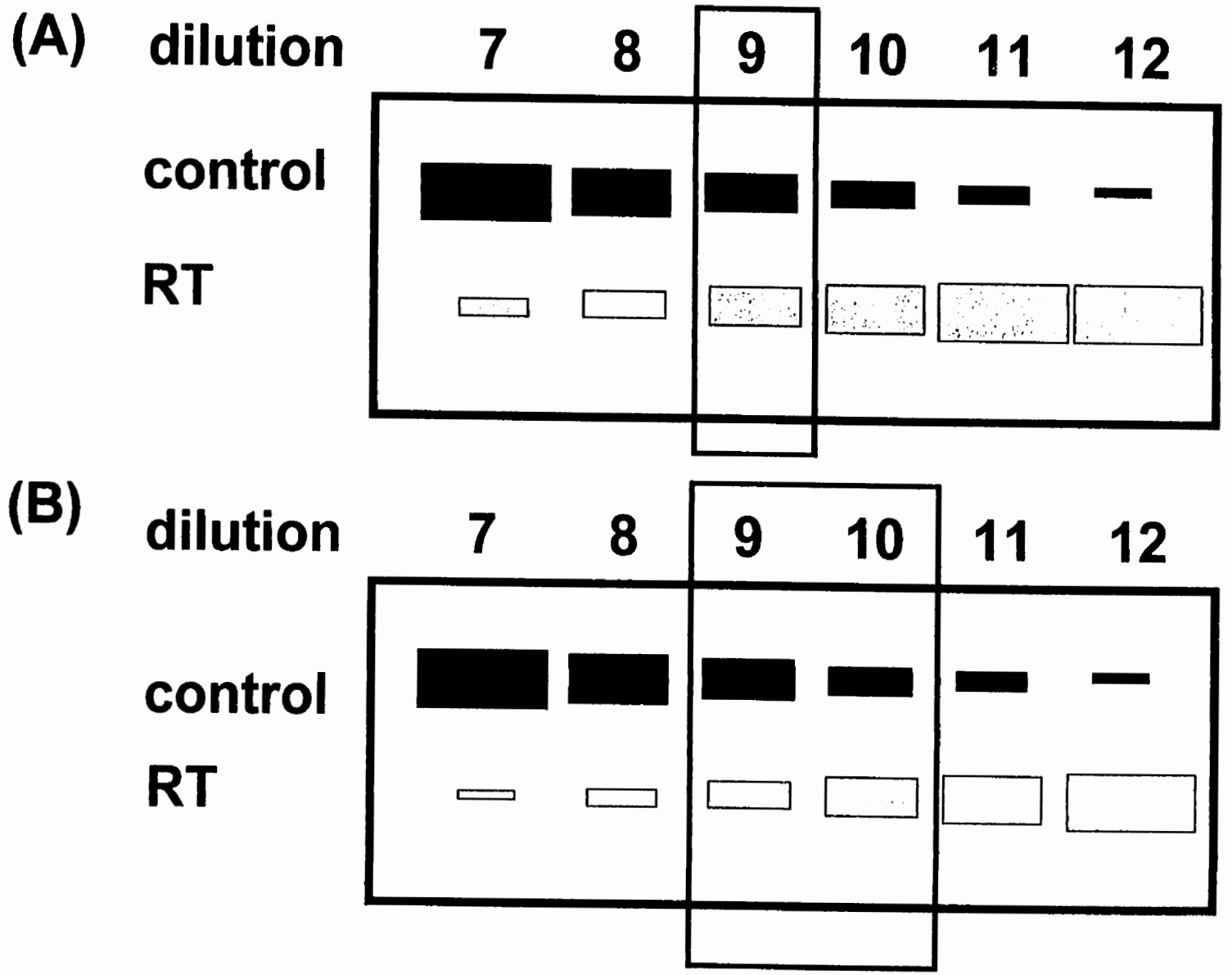
Optimal polymerase chain reaction efficiency

For optimal PCR efficiency, *G0S* gene-specific primers were selected that were separated by approximately 200 bp. The purpose of this selection was to ensure that the amplicons arising from control constructs (with exogenous DNA) did not exceed 450 bp. We verified, using ^{32}P-dATP incorporation, that both control and reverse transcribed mRNA amplicons were produced with similar efficiencies and that the number of PCR cycles (30) was optimized so that both products were made exponentially.

Calculation of mRNA levels

Competitive PCR profiles are schematically represented (Fig. 2-1). We define the "equivalence point" as the dilution in which the intensity of the control band on an agarose

Figure 2-1. **Schematic representation of equivalence point determination using RT-competitive PCR.** Schematic diagram illustrating interpretation of typical competitive analysis profiles in which the equivalence point falls *at* a particular control dilution (A: i.e. equivalence point=9) or *between* two adjacent dilutions (B: equivalence point=9.5). Results are as determined by visual inspection of EtBr intensities. RT refers to PCR products arising from the reverse transcribed RNA samples, whereas control refers to those arising from *G0S* gene cDNA control.


(A)
dilution
7
8
9
10
11
12
control
RT
(B)
dilution
7
8
9
10
11
12
control
RT

gel, stained with ethidium bromide, is equal to that of the cDNA. Equivalence points were noted either *at* a particular control dilution (Fig. 2-1A; equivalence point = 9) or *between* two control dilutions (Fig. 2-1B; equivalent point = 9.5).

A value for the number of RNA (molecules/μg total RNA) was then calculated as follows: two-fold dilutions of control DNA were carried out (starting from number 1) decreasing as the numbers increase (i.e. control DNA in dilution 10 is half as much as in dilution 9). The concentration of control DNA in dilution 1 is 8.54×10^7 molecules/μl. This value is calculated as follows:

length of control template = 5338 bp

Molecular weight = (5338 bp) x (660 g/mol bp) = 3.52×10^6 g/mol

concentration of dilution 1 = 0.5 ng/µl

molecules =(0.5×10^{-9} g/ 3.52×10^6 g/mol) (6.02×10^{23} molecules/mol)

= 8.5×10^7 (corresponds to 1 µl)

In the competitive profile shown in Fig. 2-1A, the equivalence point (= control dilution 9) is calculated as follows:

molecules/μg RNA

=[((8.5×10^7 molecules/μl x 5.0 μl)/2^{e-f})/ ((0.25 μg total RNA/165 μl) x 5.0 μl)] x a x b

= 6.6×10^8 molecules/μg RNA

a = 2 , correction for strand number (controls are double-stranded while cDNA are single-stranded)

b = 1.5, correction for size of amplicon assuming EtBr-staining directly proportional to DNA length

e = equivalence point control dilution (i.e. = 9)

f = first control dilution in the series (1)

Statistics

Data values are presented with standard errors of the mean (SEMs) since these are what we were usually interested in (i.e. if we were to repeat the experimental series, within what range might we expect the mean to occur). The significance of differences between sample means was calculated using a 2-tailed t-test ($t = x-\mu / s/\sqrt{n-1}$, where n = sample number, x = observed mean, μ = hypothetical value, s = standard deviation). The method of paired comparisons was implemented to test as Null Hypothesis the assumption that the levels of *G0S* gene mRNA in test samples and control samples are equal (i.e. $\mu = 0$). These are standard procedures described in elementary statistics texts (109, 110). We have also adopted the procedure widely employed in the scientific literature, of usually showing the results of a representative experiment with the relevant statistics for the corresponding series of experiments in the legend or text.

Alignments

Protein and nucleic acid sequence alignments were carried out using CLUSTAL (111) and GENALIGN (Oxford Molecular, Campell, CA). Predicted transmembrane segments were obtained using TMPRED (112). This, and the protein motif package PROSITE, were accessed through the ExPASy World Wide Web server (113).

RESULTS

Preincubation lowers G0S2 RNA levels

The *G0S2* gene was identified by differential RNA expression in freshly prepared blood mononuclear cell cultures treated with Con-A and cycloheximide for 2 hr. Consistent RNA increases, in response either to Con-A or to cycloheximide, were demonstrated by conventional RNA blotting (6, 27). Later, we found that basal levels of expression of *G0S* genes, and responses to inducing agents, varied depending on whether blood mononuclear cells were freshly prepared from a human donor, or had been preincubated for 24 hr (28, 35). Table 2-1 shows that, as in the case of RNAs corresponding to the "immediate-early" genes *G0S30/EGR1*, *G0S7/FOS* and *G0S3/FOSB*, a preincubation for 24 hr decreases the RNA levels of *G0S2*.

Response to Con-A depends on preincubation time

Elevated levels of various RNA species in freshly prepared cells is attributed to some stimulatory effect of the isolation procedure since basal levels increase transiently during the first hour of culture, a response which is less apparent in the case of preincubated cells (28). These "spontaneous" transient increases in the RNA levels of *G0S2*, *G0S30/EGR1*, *G0S7/FOS* and *G0S3/FOSB* occur in the absence of added stimulants and tend to mask the response to Con-A. This is particularly evident in freshly isolated cells when responses are highly variable (Figs. 2-2a, 2-2b, 2-2c, 2-2d). Responses to Con-A are therefore best demonstrated with cells which have been preincubated for 24 hr (Figs. 2-2e, 2-2f, 2-2g, 2-2h). At this time *G0S2* RNA levels increase in response to Con-A peaking at 1-2 hr after

TABLE 2-1. **RNA LEVELS[a] IN FRESHLY ISOLATED AND INCUBATED CELLS**

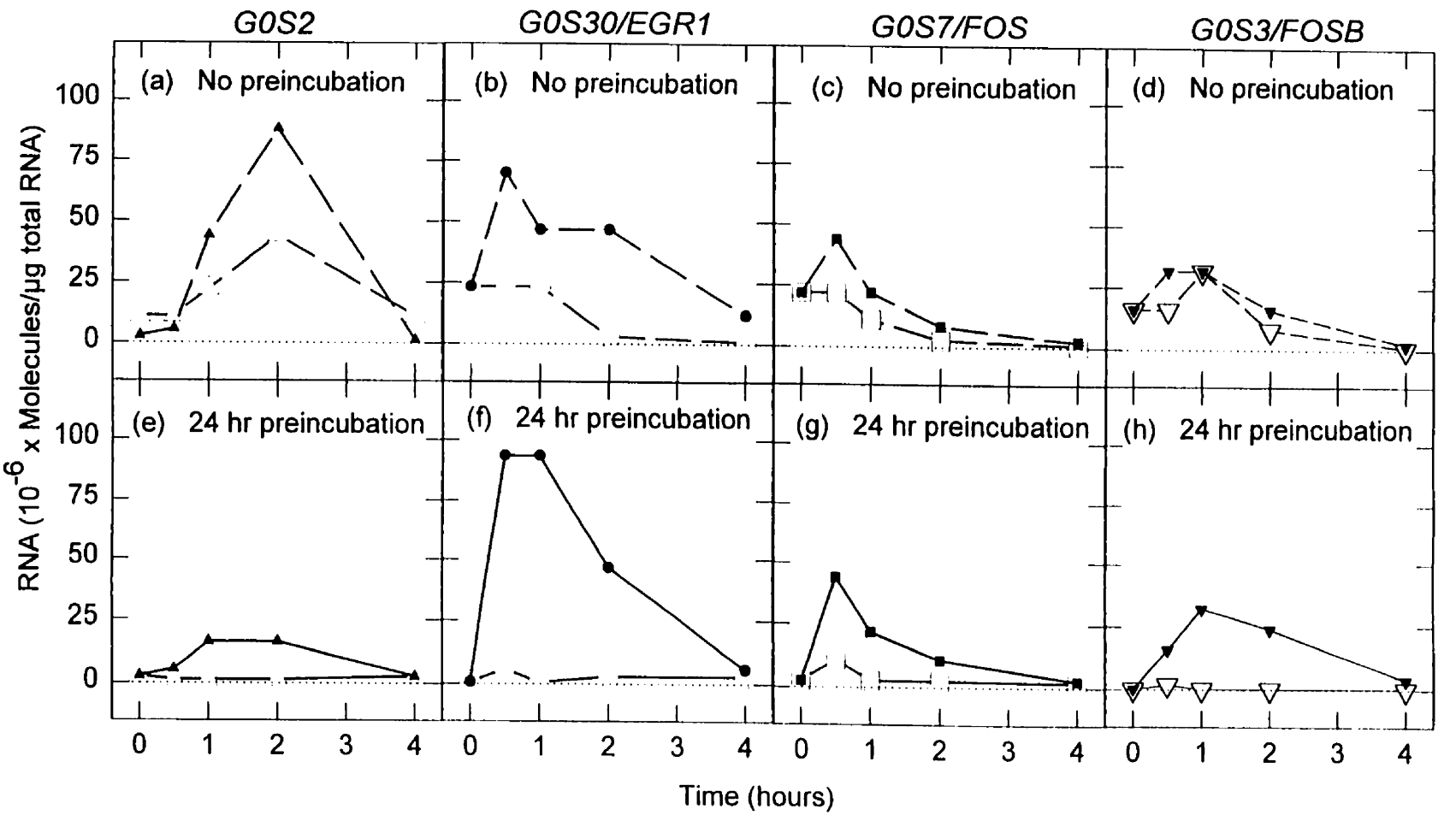
Gene	TIME		CHANGE (%)[b]	P[b]
	0 hr	24 hr		
G0S2	25.7 ± 9.7 (6)	7.0 ± 1.4 (17)	-77.7 ± 6.2 (6)	<0.001
EGR1/G0S30	19.5 ± 6.4 (6)	2.2 ± 1.0 (21)	-91.6 ± 3.7 (6)	<0.001
FOS/G0S7	37.4 ± 8.4 (6)	4.3 ± 1.0 (21)	-88.7 ± 3.0 (6)	<0.01
FOSB/G0S3	41.2 ± 21.7 (7)	1.1 ± 0.5 (16)[c]	< -98.0 (7)	<0.01

[a] 10^{-6} x [Molecules/μg total RNA] with SEM. The numbers of experiments are in parentheses. An experiment involves cells from a distinct donor that are cultured on a distinct occasion for 0 hr and/or 24 hr. At these times RNA is prepared and RNA-specific primers are added. After reverse transcription, separate aliquots are subjected to PCR amplification with RNA-specific primer pairs.

[b] Calculations were performed on six, or seven (*G0S3/FOSB*) paired values obtained from individual experiments.

[c] 16 out of 24 experiments. In the remaining 8 experiments *G0S3/FOSB* levels were undetectable (<0.2 x 10^6 molecules/μg RNA).

Figure 2-2. **Comparison of the effects of Con-A on levels of RNAs corresponding to *G0S2* (a, e), *G0S30/EGR1* (b, f), *G0S7/FOS* (c, g), and *G0S3/FOSB* (d, h) in cultured human blood mononuclear cells.** Figure illustrates one representative experiment. Filled symbols indicate cultures with Con-A (200 μg/ml). Open symbols indicate cultures without Con-A. RNA was prepared at the indicated times and subjected to RT-PCR analysis. For freshly explanted cells (a, b, c, d), the average increase in RNA levels (10^{-6} × molecules/μg RNA), relative to control cultures, were 14.7±14.7 (*G0S2* at 2 hr; P=0.4, 3 experiments). 12.8±8.8 (*G0S30/EGR1* at 30 min; P=0.2, 5 experiments), 15.0±7.5 (*G0S7/FOS* at 30 min; P=0.2, 3 experiments), and 5.2±5.9 *(G0S3/FOSB* at 60 min; P=0.4, 4 experiments). For cells preincubated for 24 hr (e, f, g, h), corresponding values were 42.6±16.5 (*G0S2*; P=0.04, 7 experiments), 235.5±99.8 (*G0S30/EGR1*; P=0.04, 10 experiments), 33.2±12.6 (*G0S7/FOS*; P=0.04, 7 experiments), and 16.9±5.0 (*G0S3/FOSB*; P=0.01, 8 experiments). P values refer to the probability that the value is not significantly different from zero.


G0S2
G0S30/EGR1
G0S7/FOS
G0S3/FOSB
(a) No preincubation
(b) No preincubation
(c) No preincubation
(d) No preincubation
(e) 24 hr preincubation
(f) 24 hr preincubation
(g) 24 hr preincubation
(h) 24 hr preincubation
RNA (10-6 x Molecules/µg total RNA)
100
75
50
25
0
0 1 2 3 4
Time (hours)

lectin addition and then fall to baseline levels by 4 hr. RNA levels peak sooner in the cases of *G0S30/EGR1*, and *G0S7/FOS* and slightly sooner in the case of *G0S3/FOSB*.

Response to cycloheximide depends on preincubation time

G0S gene RNA levels increase in response to cycloheximide in an approximately rectilinear fashion over the 45 min period studied, but the data were only significant for freshly explanted cells in the case of *G0S7/FOS* ($P<0.05$; Fig. 2-3). All the *G0S* genes responded in preincubated cells. However, the response of *G0S2* was not significant. The *G0S3/FOSB* response in preincubated cells was small, but significant ($P<0.05$).

Response to TPA and ionomycin, separately, and in combination

TPA and ionomycin rapidly enter cells and appear to by-pass the cell surface receptors involved in transmembrane signalling by antigen or lectin (89). When TPA alone is added to cells which have been preincubated for 24 hr, *G0S2* RNA levels increase, peaking at 30 min, but the increase is of marginal significance (Table 2-2). The increases of the other *G0S* gene RNAs studied are significant, especially *G0S30/EGR1* RNA.

G0S2 RNA levels increase in response to ionomycin alone and peak at 60 min, but the increase is not significant ($P=0.330$). Levels of *G0S3/FOSB* RNA also increase after ionomycin addition, peaking at 60 min ($P=0.015$). *G0S7/FOS* RNA increases in response to ionomycin addition, but is not significant either at 30 min ($P=0.10$) or at 60 min ($P=0.073$) following treatment. In contrast to the response to TPA, *G0S30/EGR1* RNA increases only slightly in response to ionomycin (Table 2-2).

Figure 2-3. **Effects of the protein synthesis inhibitor cycloheximide on levels of RNAs corresponding to *G0S2*, *G0S30/EGR1*, *G0S7/FOS*, and *G0S3/FOSB* in human blood mononuclear cells.** Figure illustrates one representative experiment. Filled symbols indicate cultures with cycloheximide (100μM). Open symbols indicate cultures without cycloheximide. Details are as in Figure 2-2. For freshly explanted cells (a, b, c, d) the average increases in RNA levels (10^{-6} × molecules/μg RNA) relative to controls at 45 min, were 132.0 ± 66.2 (*G0S2*; *P*=0.2), 119.8 ± 32.1 (*G0S30/EGR1*; *P*=0.07), 292.7 ± 59.1 (*G0S7/FOS*; *P*=0.04), and 259.3 ± 121.5 (*G0S3/FOSB*; *P*=0.2). For cells preincubated for 24 hr (e, f, g, h) corresponding values were 82.5 ± 44.0 (*G0S2*; *P*=0.2), 42.5 ± 3.9 (*G0S30/EGR1*; *P*=0.01), 70.0 ± 13.9 (*G0S7/FOS*; *P*=0.04), and 13.0 ± 2.6 (*G0S3/FOSB*; *P*=0.04). These data refer to 3 independent experiments performed with cells from different donors on different occasions.

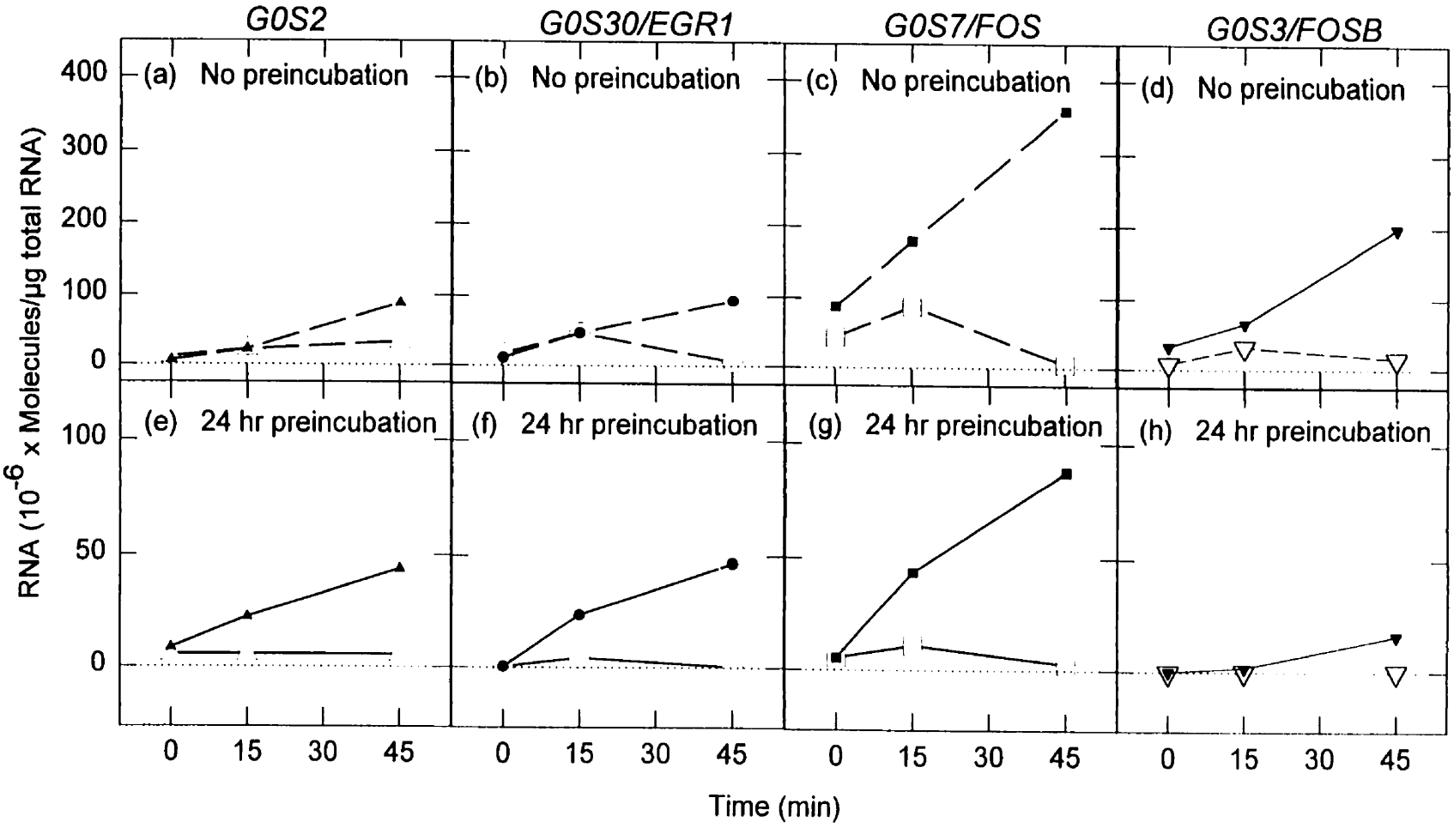

G0S2
G0S30/EGR1
G0S7/FOS
G0S3/FOSB
(a) No preincubation
(b) No preincubation
(c) No preincubation
(d) No preincubation
(e) 24 hr preincubation
(f) 24 hr preincubation
(g) 24 hr preincubation
(h) 24 hr preincubation
RNA (10-6 x Molecules/µg total RNA)
400
300
200
100
0
100
50
0
0
15
30
45
Time (min)

TABLE 2-2. **CHANGES IN RNA LEVELS[a] IN RESPONSE TO TPA AND IONOMYCIN, EITHER SEPARATLY, OR IN COMBINATION**

Gene	Time (min)	CONTROLS	TPA	P^b	IONOMYCIN	P^b	TPA + IONOMYCIN	P^b
G0S2	30	12.2 ± 4.3 (5)	34.4 ± 17.9 (4)	0.160	4.5 ± 1.0 (4)	0.920	18.9 ± 4.6 (7)	0.160
	60	9.4 ± 3.8 (5)	14.4 ± 4.7 (4)	0.220	11.9 ± 3.8 (4)	0.330	94.7 ± 30.8 (7)	0.017
G0S30/EGR1	30	1.4 ± 1.1 (5)	225 ± 38.3 (4)	0.005	6.4 ± 2.8 (4)	0.098	187 ± 86 (7)	0.036
	60	0.8 ± 0.5 (5)	122 ± 45.6 (4)	0.038	5.7 ± 1.2 (4)	0.010	127 ± 32 (7)	0.004
G0S7/FOS	30	5.2 ± 2.2 (6)	47.8 ± 10.9 (5)	0.009	30.2 ± 16.4 (5)	0.100	83.7 ± 27.2 (8)	0.012
	60	4.1 ± 0.9 (6)	18.1 ± 7.7 (5)	0.072	25.8 ± 12.0 (5)	0.073	70.9 ± 29.4 (8)	0.029
G0S3/FOSB	30	0.7 ± 0.2 (6)	20.2 ± 7.6 (5)	0.031	29.0 ± 6.9 (5)	0.008	163 ± 115 (8)	0.100
	60	1.3 ± 0.6 (6)	18.6 ± 7.0 (5)	0.034	35.5 ± 10.4 (5)	0.015	112 ± 55 (8)	0.041

[a] 10^{-6} x [Molecules/μg total RNA] with SEM. The number of experiments are in parentheses.

[b] *P* values are determined for increases in RNA levels in TPA-, Ionomycin-, and TPA+Ionomycin-treated cultures relative to RNA levels in control cultures using the method of paired comparisons as outlined in Materials and Methods.

At 60min in cultures treated with both TPA and ionomycin levels of *G0S2* RNA are significantly greater than levels induced by TPA (*P*=0.021)or by ionomycin alone (*P*=0.019). Similar respective *P* values for *G0S30/EGR1* (30 min) are 0.650 and 0.039, for *G0S7/FOS* (30 min) are 0.130 and 0.062, and for *FOSB/G0S3* (60 min) are 0.066 and 0.100.

The combination of TPA and ionomycin greatly increases absolute levels of *G0S2* RNA (peaking at 1 hr), and of *G0S3/FOSB RNA*. The response is synergistic, in the sense that it is greater than the sum of the responses produced by the agents separately (Table 2-2). A tendency to synergism is also noted in the case of *G0S7/FOS RNA*. On the other hand, ionomycin has only a minor influence on levels of *G0S30/EGR1* RNA, either alone, or in combination with TPA. The results indicate that the response of *G0S30/EGR1* is mainly due to TPA (hence presumably mediated by protein kinase C), whereas the responses of *G0S2*, *G0S3/FOSB*, and *G0S7/FOS* show decreasing degrees of synergism between TPA and ionomycin (implicating calcium as a major influence on *G0S2* expression).

CsA inhibits the increase of G0S2 mRNA by TPA + ionomycin and by Con-A

In the 5' flank of the *G0S2* and *G0S3/FOSB* genes there are potential transcription factor binding sites, including an NFAT site, similar to those found in the promoters of *IL-2*, *GM-CSF/IL3*, and *IL-4* (Fig.2-4). This suggested that *G0S2* and *G0S3/FOSB* expression may be regulated by NFAT, whose transport to the nucleus is both calcium-dependent and CsA-sensitive. Indeed, preincubation with CsA for 1 hr inhibits the increase of *G0S2* mRNA both in response to TPA+ionomycin (Fig.2-5) and to Con-A (Fig.2-6). The effects of CsA were consistent for *G0S2* with average changes at 60 min of -67.0 ± 10.4% relative to cells treated only with TPA+ionomycin (6 experiments, P=0.001), and -41.7 ± 12.4% relative to cells only treated with Con-A (5 experiments, P=0.03). The inhibition of the response to TPA+ionomycin was not significantly greater than the inhibition of the response to Con-A (P>0.05). Although not clearly illustrated in the experiment shown in Fig. 2-5, CsA

Figure 2-4. **Comparison of the 5' flank of *G0S2* and *G0S3/FOSB* with 5' promoter/enhancer sequences of various NFAT-responsive genes.** Motifs for transcription factors NFAT, AP-1, and OCT (89) are underlined.

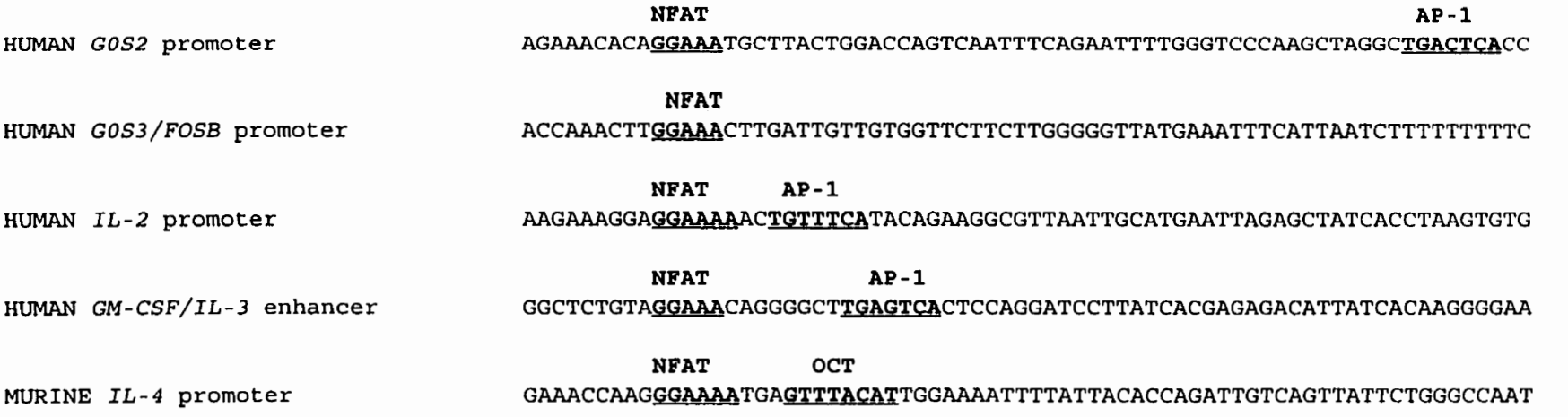

NFAT
AP-1
HUMAN G0S2 promoter
AGAAACACAGGAAATGCTTACTGGACCAGTCAATTTCAGAATTTTGGGTCCCAAGCTAGGCTGACTCACC
NFAT
HUMAN G0S3/FOSB promoter
ACCAAACTTGGAAACTTGATTGTTGTGGTTCTTCTTGGGGGTTATGAAATTTCATTAATCTTTTTTTTTC
NFAT
AP-1
HUMAN IL-2 promoter
AAGAAAGGAGGAAAAACTGTTTCATACAGAAGGCGTTAATTGCATGAATTAGAGCTATCACCTAAGTGTG
NFAT
AP-1
HUMAN GM-CSF/IL-3 enhancer
GGCTCTGTAGGAAACAGGGGCTTGAGTCACTCCAGGATCCTTATCACGAGAGACATTATCACAAGGGGAA
NFAT
OCT
MURINE IL-4 promoter
GAAACCAAGGGAAAATGAGTTTACATTGGAAAATTTTATTACACCAGATTGTCAGTTATTCTGGGCCAAT

Figure 2-5. **Effects of CsA on TPA/ionomycin-induced increases in RNA levels of *G0S2*, *G0S30/EGR1*, *G0S7/FOS* and *G0S3/FOSB* in cells preincubated for 24 hr.** Figure illustrates one representative experiment. The upper photographs show RNA profiles for cells cultured with 100 nM TPA and 2 μM ionomycin (added at 0 hr) in the absence or presence of 100 nM CsA in ethanol (added at -1 hr; i. e. cells were preincubated with CsA for 1 hr before adding TPA and ionomycin). The upper band indicates the PCR product of control cDNA template whereas the bottom band indicates the PCR product of reverse transcribed template. Graphs (lower) show the corresponding mRNA levels with filled symbols referring to cultures with CsA and open symbols referring to samples without CsA. Other details are as in Fig. 2-2. Average percentage changes in levels of RNA in response to CsA in six experiments were -67.0±10.4 (*G0S2* at 60 min; P=0.001), 41.7±27.1 (*G0S30/EGR1* at 30 min; P=0.2), -16.6±10.5 (*G0S7/FOS* at 30 min; P=0.2), and -2.4±22.0 (*G0S3/FOSB* at 60 min; P=0.9).

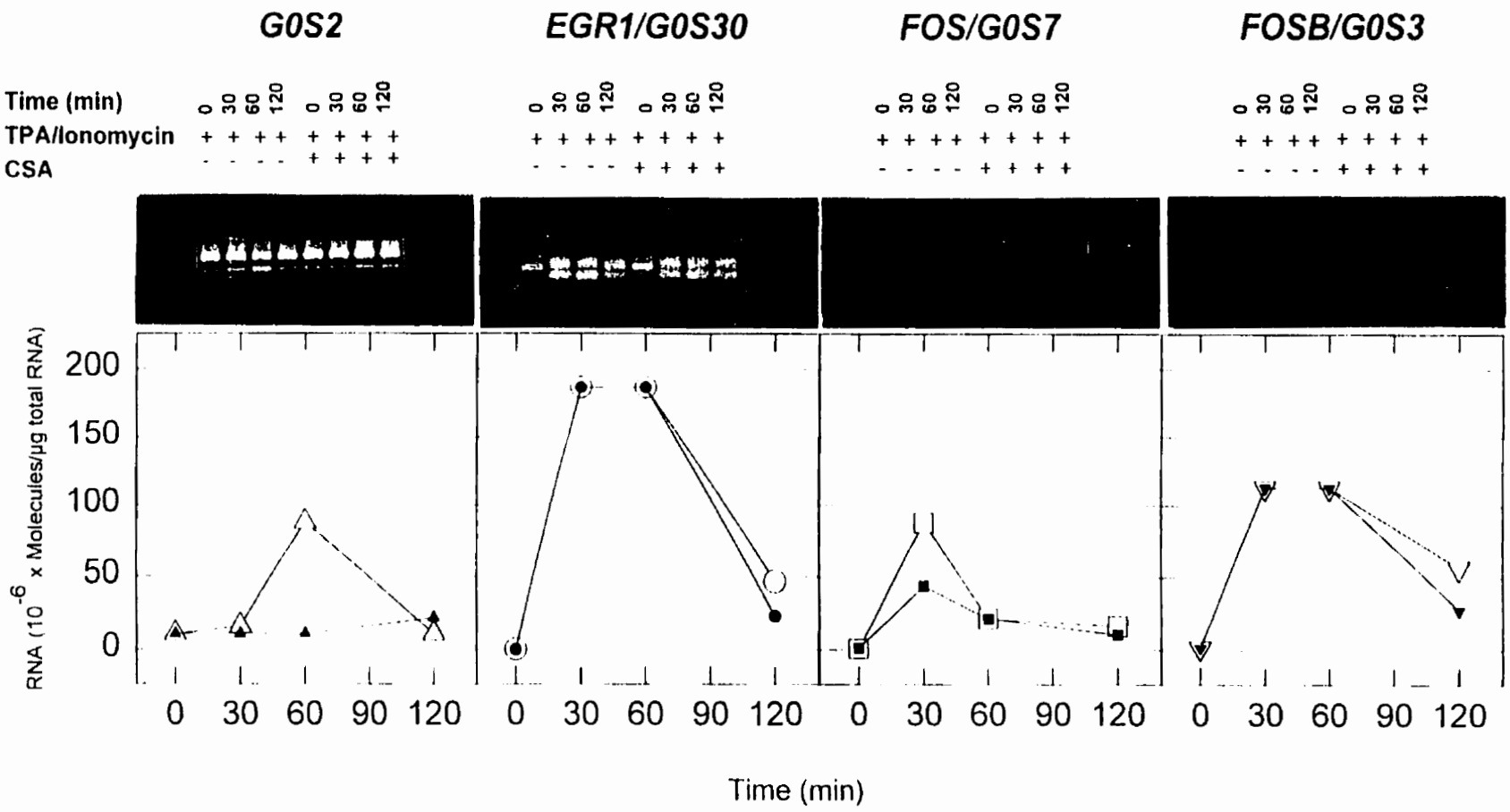
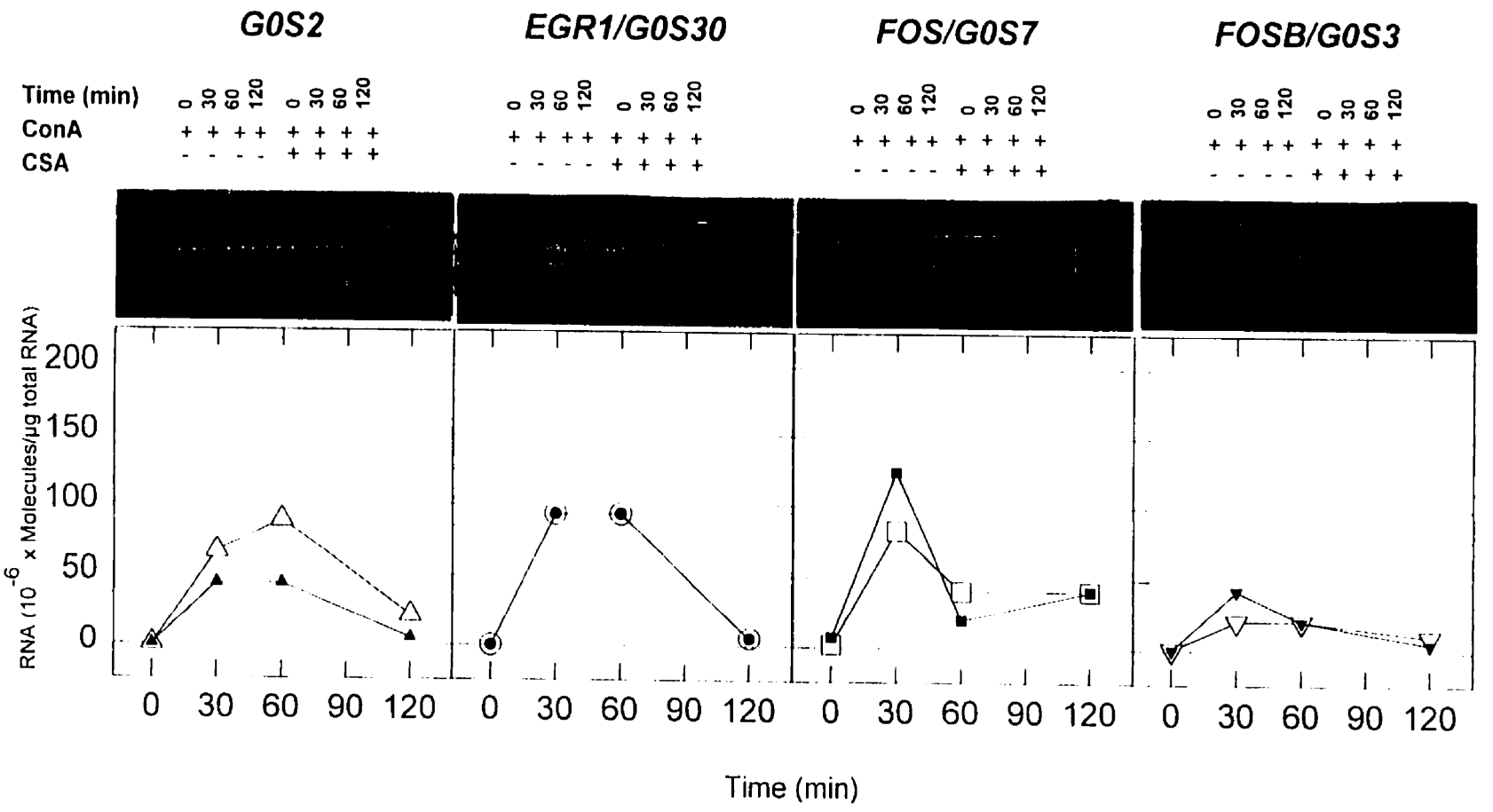

G0S2
EGR1/G0S30
FOS/G0S7
FOSB/G0S3
Time (min)
TPA/Ionomycin
CSA
0 30 60 120 0 30 60 120
+ + + + + + + +
- - - - + + + +
RNA (10-6 x Molecules/µg total RNA)
200
150
100
50
0
0 30 60 90 120
Time (min)

Figure 2-6. **Effects of CsA on Con-A-induced mRNA levels of *G0S2*, *G0S30/EGR1*, *G0S7/FOS* and *G0S3/FOSB* in cells preincubated for 24 hr.** Figure illustrates one representative experiment. The upper photographs show mRNA profiles for cells cultured with 200 μg/mL Con-A and cells treated with 100 nM CsA in ethanol for 1 hr prior to treatment with Con-A. The upper band indicates the PCR product of control cDNA template whereas the bottom band indicates the PCR product of reverse transcribed template. Graphs (lower) show the corresponding mRNA levels determined using the competitive PCR assay, for CsA/Con-A-treated cultures (filled symbols), and Con-A-treated cultures (open symbols). Other details are as in Figure 2-2. Average percent changes in levels of RNA in response to CsA, in five experiments, were -41.7±12.4 (*G0S2* at 60 min; P=0.03), -34.7±18.4 (*G0S30/EGR1* at 30 min; P=0.1), 1.3±34.5 (*G0S7/FOS* at 30 min; P=1.0), and, in four experiments, 16.6±28.9 (*G0S3/FOSB* at 60 min; P=0.6).


G0S2
EGR1/G0S30
FOS/G0S7
FOSB/G0S3
Time (min)
ConA
CSA
RNA (10-6 x Molecules/µg total RNA)
200
150
100
50
0
0 30 60 90 120
Time (min)

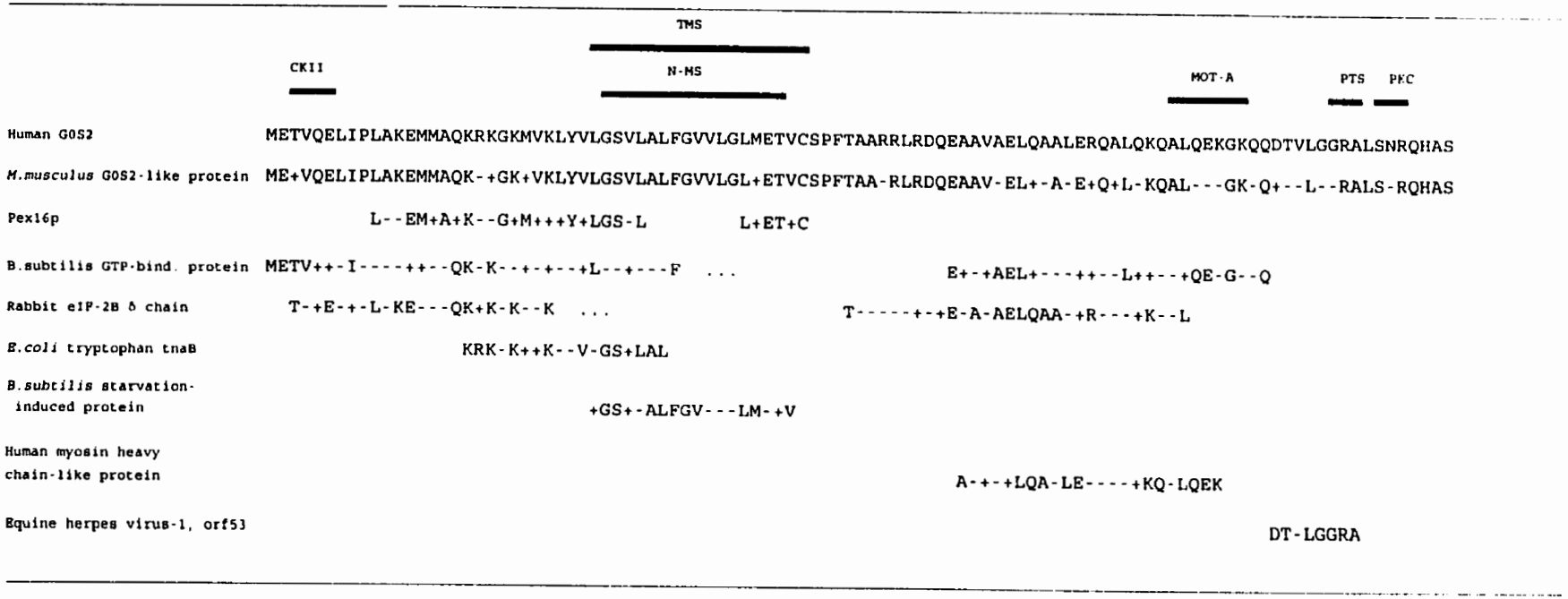
preincubation was also found to inhibit the increase of *G0S3/FOSB* mRNA in response to TPA + ionomycin. The average change at 120 min was -34.2 ± 11.5% relative to cells only treated with TPA + ionomycin (6 experiments, P=0.03). Unlike *G0S2* however, the increase in *G0S3/FOSB* mRNA in response to Con-A was not inhibited by CsA. CsA preincubation was found to have no effects on the expression of *G0S30/EGR1*, and *G0S7/FOS* RNAs.

Further sequence analysis of G0S2

Since our original GenBank search (27), a homologous mouse cDNA sequence has become available (*MMG0S2*; GenBank accession number X95280). The protein, which shares 78% amino acid identity with G0S2 (Fig. 2-7), is induced in mesenchymal progenitor cells by human bone morphogenic protein (58). Consistent with this, *G0S2* has a CpG island indicating expression in the germ line, and expressed sequence tag studies indicate expression of *G0S2* mRNA in human embryo and ovary (114).

The protein contains potential sites for phosphorylation by casein kinase II and protein kinase C, for N-myristoylation, for peroxisome targeting, and for ATP/GTP (motif A). The TMPRED program indicates the presence of a putative transmembrane segment. There are short regions of similarity with various proteins, including *Yarrowia lipolytica* Pex16p, a *Bacillus subtilis* GTP-binding protein and the δ chain of rabbit eIF-2B, which is a component of the GDP/GTP nucleotide exchange factor for protein initiation factor eIf-2 (115; Fig.2-7).

Figure 2-7. **Protein sequence alignment of G0S2 and similar proteins**. Dark horizontal lines refer to a potential casein kinase II phosphorylation site (CKII; motif [S or T]-XX-[D or E], a potential N-myristoylation site (N-MS; motif G-{none of EDRKHPFYW}-XX-[one of STAGCN]-{not P}), a potential ATP/GTP binding site (MOT-A; motif A= [A or G]-XXXX-G-K-[S or T]), a potential peroxisome targeting signal (PTS; motif [one of SAGC N]-[one of RKH]-[one of LIVMAF]), and a potential protein kinase C phosphorylation site (PKC; motif [S or T]-X-[R or K]). TMS notes a potential transmembrane segment with the N-terminus outside. The numbers in parentheses indicate the amino acid position of the match. Three consecutive periods (...) separate two regions of the same protein sequence that were found to match G0S2. A '+' symbol in the alignment indicates a similar amino acid, whereas a blank space indicates a mismatched amino acid.


TMS
CKII
N-MS
MOT·A
PTS
PKC
Human G0S2
METVQELIPLAKEMMAQKRKGKMVKLYVLGSVLALFGVVLGLMETVCSPFTAARRLRDQEAAVAELQAALERQALQKQALQEKGKQQDTVLGGRALSNRQHAS
M.musculus G0S2-like protein
ME+VQELIPLAKEMMAQK-+GK+VKLYVLGSVLALFGVVLGL+ETVCSPFTAA-RLRDQEAAV-EL+-A-E+Q+L-KQAL---GK-Q+--L--RALS-RQHAS
Pex16p
L--EM+A+K--G+M+++Y+LGS-L
L+ET+C
B.subtilis GTP-bind. protein
METV++-I----++--QK-K--+-+--+L--+---F
E+-+AEL+---++--L++--+QE-G--Q
Rabbit eIF-2B δ chain
T-+E-+-L-KE---QK+K-K--K
T-----+-+E-A-AELQAA-+R---+K--L
E.coli tryptophan tnaB
KRK-K++K--V-GS+LAL
B.subtilis starvation-induced protein
+GS+-ALFGV---LM-+V
Human myosin heavy chain-like protein
A-+-+LQA-LE----+KQ-LQEK
Equine herpes virus-1, orf53
DT-LGGRA

DISCUSSION

Primary action of CsA during the G_0/G_1 switch

CsA decreases *IL2* gene transcription and synthesis of IL2, which is involved in progression through the G_1 phase of the cell cycle (68). It is believed that "the ability of...CsA to block T cell activation can be overcome by the addition of IL2 to cells, demonstrating that the inhibition of IL2 production is crucial to [its] mode of action" (69). Furthermore, it was recently stated as a "fact", that "CsA-mediated inhibition of T cell activation can be overcome by addition of exogenous IL2" (116). However, there is abundant evidence from a variety of systems that inhibition by CsA of lymphocyte progression through the G_1 phase and entry into S phase cannot be reversed by IL2 and other lymphokines (70-72). Inhibition of *IL2* gene transcription, and a consequent inhibition of translation and secretion of IL2, may be necessary, but would not appear sufficient, for immunosuppression by CsA.

The phase of greatest CsA sensitivity occurs shortly after lymphocyte activation, when cells are becoming committed to enter the G_1 phase (93). Thus, inhibition of the G_0/G_1 switch, rather than G_1 progression, appears most important for the immunosuppressive role (95). This might involve one or more of the genes which are differentially expressed during the switch. Indeed, Gunter *et al.*, (117) reported that early RNA expression of the β-chemokine G0S19/LD78/464 was cyclosporin-sensitive. Our present results (Figs.2-5, 2-6) implicate *G0S2*, rather than the zinc finger transcription factor encoding gene *G0S30/EGR1*, and the two genes whose products are part of transcription factor complexes, *G0S7/FOS* and *G0S3/FOSB* (99).

Calcium-dependence of CsA inhibition

Studies with varying proportions of TPA and ionomycin indicate calcium dependence of inhibition (70). Indeed, the calcium and calmodulin-dependent protein phosphatase, calcineurin, is inhibited by CsA. While calcineurin may have several functions (118), an important function is the dephosphorylation of members of the NFAT family of transcription factors; this is necessary for their translocation from the cytoplasm to the nucleus, which occurs within 5 min of lymphocyte activation (101, 119).

Unlike the CsA-insensitive *G0S30/EGR1* gene, increased RNA expression of the CsA-sensitive *G0S2* gene is dependent on ionomycin (Table 2-2), suggesting calcium-dependence. Furthermore, the increase in *G0S2* RNA level occurs early (Fig.2-2), at a time when cells are becoming committed to the G_1 phase of the cell cycle. *G0S2* appears a possible NFAT target gene, and has a potential "purine box" site in the 5' flank (Fig.2-3).

Whether the increase in *G0S2* RNA is due to a change in transcription, or stabilization of an RNA which is rapidly turning over (120) is not known. An involvement of NFAT, as indicated by CsA sensitivity, would suggest that the increase is due to an increase in transcription rate. Alternative, cycloheximide might decrease the concentration of a labile repressor protein, so that the transcription rate would increase. In some systems transient inhibition of protein synthesis is itself sufficient to trigger the G_0/G_1 switch and allow G_1 progression when the inhibitor of protein synthesis is removed (121, 122).

CsA inhibits both positive and negative selection of T and B lymphocytes

While the G0S2 protein has some intriguing characteristics (Fig.2-7), its function is

not known. If it has a role in the normal lymphocyte response to antigens ("self" or "not-self"), then this might be inhibitory and/or stimulatory. The fact that *G0S2* RNA expression is inhibited by a known immunosuppressant, CsA, suggests that the role of G0S2 is stimulatory. However, since lymphocyte-ligand interactions can transmit either positive or negative signals, the possibility that G0S2 might be involved in lymphocyte activation and/or inactivation (123), is not excluded.

During their initial education, or thereafter, antigen may negatively select lymphocytes, leading to a state of immunological tolerance (124). In the case of self antigens, high specificity anti-self cells are eliminated as part of the education process. The elimination may be mediated by serum factors with some of the properties of complement (125, 126), or may require the cell-autonomous triggering of a calcium-dependent apoptotic cascade (119); this may involve "immediate-early" genes like *G0S2* (123), and can be inhibited by CsA (127-129). It should be noted that in some circumstances CsA can inhibit both complement-mediated and cell-autonomous cytotoxic effects (130).

Antigen may also positively select cells, a process which, during their initial education, is likely to involve the selection of lymphocytes with receptors of low or moderate specificity for "near-self" (125, 126), or "altered self" (131) antigenic determinants (103). This process can be simulated with TPA + ionomycin (132), and is inhibited by CsA (71, 127). When CsA is administered to experimental animals there first appears to be inhibition of negative selection so that high specificity anti-self cells have the opportunity to accumulate (133). CsA would also prevent their activation (positive selection) by self antigens. However, when CsA is withdrawn autoimmune disease is provoked (102, 134); this

implies that negative selection is restored less rapidly than positive selection. However, others report that CsA inhibits only positive selection (135). Recently Shao *et al.* (136), in an attempt to distinguish intracellular signalling pathways involved in positive and negative selection in mice, found that increased expression of *EGR1/G0S30* is insensitive to CsA, as in the present work (Figs. 2-4, 2-5). In future such studies may be assisted by well characterized reporter genes, such as *G0S2*, whose expression responds very rapidly to CsA.

Positive selection by self and not-self antigens may influence both T and B cells (103, 137-139). Similarly, both T and B cells are inhibited by CsA (140-142). In B cells positively selected by antigen, CsA prevents induction of the immediate-early *TNFα* gene, and of latent Epstein-Barr virus (EBV). As in the case of *G0S2* (Table 2-2), TPA and ionomycin act synergistically to induce latent EBV, and it is the ionomycin-dependent component of this which is inhibited by CsA (143).

Yeast homologs of mammalian cell cycle genes

There are many similarities between cell cycles of yeast, which respond to pheromones and nutritional cues, and of mammals, which respond to ligands for various cell surface receptors (144). Yeast signal transduction pathway mutants have greatly assisted identification of the corresponding signalling components in higher eukaryotes (90). Recently, similarities between genes implicated in the yeast cell cycle and mammalian *G0S* genes have emerged (Table 1-1). Thus, a homolog of *G0S30/EGR1* in budding yeast mediates glucose repression of genes whose transcription increases on starvation (145). A homolog of *G0S24/TIS11* (40) is involved in the pheromone response in fission yeast (145).

The budding yeast protein SST2, a homolog of the regulator of G protein signalling G0S8/RGS2 (31), facilitates recovery from pheromone-imposed cell cycle arrest, which may be analogous to the G_0 phase of the mammalian cell cycle (146); this switch (i.e. reentry into the G_1 phase of the cell cycle) is inhibited by CsA (91) in a calcineurin-dependent manner (97, 98).

Although the sequence of the entire genome of *S. cerevisiae* is now available, we have found no obvious *G0S2* homolog. However, initially we could only detect weak similarities between the protein sequence of *SST2* and *G0S8/RGS2*. Yet G0S8/RGS2 can functionally replace SST2 in yeast mutants (reviewed in 8). There are some sequence similarities between G0S2 and the Pex16p protein of yeast *Yarrowia lipolytica* (Fig. 2-7). Using a *Y. lipolytica* genomic DNA library, the *PEX16* gene was isolated by functional complementation of the pex16-1 mutant strain that is defective in peroxisomal assembly (147). *PEX16* encodes the peroxin Pex16p, which is a peripheral peroxisomal membrane protein with no known homologs (148). It is possible that G0S2 is a Pex16p homolog and thus, is required for peroxisomal biogenesis. Both the presence of a peroxisomal targeting signal at the C-terminus as well as a putative transmembrane segment in G0S2 (Fig. 2-6) support this hypothesis. Recent studies that investigated *G0S2* mRNA expression during murine embryonic development revealed that the mouse homolog of *G0S2* is almost exclusively expressed in adipogenic tissue (G. Gross, personal communication). Noting that peroxisomes are responsible for the β-oxidation of fatty acids (149), and breakdown of hydrogen peroxide (150), the aforementioned finding further supports the suggestion that G0S2 may serve a crucial role in peroxisomal assembly. In future studies, localization

studies will help us to assess this potential role, using an anti-G0S2 antibody. This will allow us to assess the hypothesized absence of G0S2 in human cell lines defective in peroxisomal assembly. Future studies examining the ability of G0S2 to functionally replace Pex16p in *Y. lipolytica* mutant strains will further test this potential role of G0S2.

Inability to Express G0S2 protein In E. coli

In order to help us gain an understanding of both the structure and function of G0S2, a project was undertaken to express the G0S2 protein (See Appendix A1). G0S2 expression would be of great value for raising antibodies for future localization studies. This antisera would also be instrumental in examining G0S2 protein levels under various conditions, thus allowing us to correlate protein level increase with that of mRNA levels. It would enable us to test if the decrease in *G0S2* mRNA noted correlates with a decrease in protein levels. Moreover, it would enable us to assay the extent of G0S2 inhibition in antisense oligonucleotide studies. This work would enable us to further characterize G0S2 and decipher its function. The expressed protein would also allow us to commence crystallization studies which could enable us to elucidate the structure of G0S2.

Several attempts at expressing recombinant G0S2 protein proved unsuccessful. One reason for the lack of expression may be that G0S2 utilizes codons that are less frequently used by *E. coli*. A previous study using the Genetics Computer Group program Codon Preference revealed the enrichment of human codons in the second exon of *G0S2*, which is consistent with this hypothesis (27). Re-engineering the *G0S2* cDNA sequence to maximize usage of codons frequently used by *E. coli* may facilitate G0S2 protein expression. Another

possible reason for the lack of G0S2 expression may be the presence of a hydrophobic region, particularly its transmembrane segment (Fig. 2-6). Such regions often exhibit a toxic effect on host cells, likely due to an association of the protein with crucial membrane systems (151). The removal of this region may also facilitate G0S2 protein expression in future studies. The inability to express G0S2 protein may also reflect G0S2 instability in *E. coli*. This is generally found with short protein sequences that are quickly degraded by cellular proteases, although the selected BL21 strain is deficient in the Ion and ompT proteases. Often, a reduction in growth temperature and induction time can overcome this instability, yet both had no effect on the ability to express G0S2 protein.

CHAPTER 3

A "Stealth" Approach to Inhibition of Lymphocyte Activation by Oligonucleotide Complementary to the Putative G_0/G_1 Switch Regulatory Gene *G0S30/EGR1/NGFI-A*.

ABSTRACT

The putative G_0/G_1 switch regulatory gene *G0S30/EGR1/NGFI-A* shows increased expression shortly after adding concanavalin-A (Con-A) to cultured T lymphocytes. However, it is reported that lymphocytes from mice in which the gene has been deleted proliferate normally in response to Con-A. This suggests that *G0S30* expression is not critical for the response. Paradoxically, there is also a report that proliferation of Con-A-stimulated rat lymphocytes is inhibited by an antisense oligonucleotide complementary to *G0S30*. Because the *G0S30* sequence is highly conserved between species, we used a similar oligonucleotide (differing by 1 base) to show for humans that the response to Con-A is also inhibited. However, no oligonucleotide-induced changes in the concentrations of *G0S30* protein or mRNA are detectable. This suggests that the oligonucleotide is not acting by influencing the expression of *G0S30*, and may be affecting the expression of another gene. The phosphorothioated oligonucleotide was maximally inhibitory at a 50 nM concentration, which is near to the "physiological" concentration at which CpG-containing oligonucleotides are reported to activate mouse B lymphocytes. In the present work, increasing the concentration above 50 nM, or adding further quantities of control oligonucleotides, decreased the inhibition. It is suggested that by using low oligonucleotide concentrations, one may avoid "tripping" an endogenous defense system directed against exogenous

oligonucleotides, yet still get sufficient uptake to inhibit lymphocyte activation.

INTRODUCTION

The observation that mRNA levels of *G0S* genes increase rapidly after human lymphocytes are treated with Con-A suggests but does not prove that these genes play an important role in the G_0/G_1 switch. Ideally, one would like to inhibit the expression of a particular *G0S* gene and examine what effects this would have on lymphocyte activation. To accomplish this one could employ antisense oligonucleotides, which are able to enter cultured cells and inhibit the expression of specific genes (152), including leukemia-specific genes (153, 154), and genes of intracellular pathogens (155-158). However, unless artificial methods to facilitate entry into cells are employed (159), even phosphorothioated oligonucleotides are required at concentrations greater than 1 μM. Why are such relatively high concentrations of oligonucleotides necessary to inhibit the expression of target genes?

Nucleic acids being major cell constituents and cell death being a ubiquitous phenomenon, it is likely that early in evolution unicellular organisms became adapted to being exposed intermittently to the oligonucleotide nucleic acid degradation products of other organisms. At low concentrations, these products would have been of nutrient value and appropriate receptors and channels for degradation would have evolved. At high concentrations, the possibility of access to compartments where the oligonucleotides could affect the functioning of the cell would arise. Indeed, for several hours after feeding M13 phage to mice phage, DNA fragments as long as 976 nucleotides may be found in white blood cells (160). Thus, there would have been an evolutionary pressure for the development

of monitoring and defense systems against foreign DNA and its partial degradation products.

The recognition of a nucleic acid as foreign, and the initiation of an appropriate adaptive response, has clear precedents in mammalian systems. Exogenous double-stranded RNAs are known to trigger production and activation of interferon and RNase L (161). Bacterial DNA or exogenous single-stranded oligonucleotides induce the proliferation of murine B lymphocytes (162). This has been related to the presence of unmethylated CpG dinucleotides, which might alert B lymphocytes to the presence of pathogenic microorganisms (163). Thus, oligonucleotide recognition can be a *physiological process*. Oligonucleotides containing unmethylated CpG residues are active at 30 μM concentration, and the corresponding oligonucleotides phosphorothioated to increase stability are active at only a 30 nM concentration.

We have explored the hypothesis that, above a critical concentration, oligonucleotides "trip" an endogenous defense system directed against exogenous oligonucleotides. The need to overwhelm this system to achieve specific inhibition of gene expression would explain why high oligonucleotide concentrations are normally required. Alternatively, the concentration might be lowered to avoid "tripping" the system. In view of possible therapeutic applications, we examined the use of very low concentrations of oligonucleotides with cultured human blood lymphocytes activated to enlarge and proliferate by the lectin Con-A. Since Perez-Castillo and co-workers (164) had shown inhibition of lymphocyte proliferation in Con-A-activated rat lymphocytes by an antisense oligonucleotide directed against the *G0S30/EGR1/NGFI-A* gene, we chose to study this *G0S* gene initially. However, while the work was in progress, Lee and co-workers (165) reported that,

paradoxically, Con-A-induced lymphocyte proliferation is *not* impaired in mice when both copies of the *G0S30* gene are deleted. Our studies therefore address both this paradox as well as the endogenous defense system hypothesis.

MATERIALS AND METHODS

Phosphorothioated oligonucleotides

To reduce the possibility of artifacts due to contaminants, oligonucleotides were obtained from independent suppliers each using different purification methods. These were the DNA Service, Queen's University, and BioSynthesis Inc., Texas. In the former case, purification was by thin-layer chromatography and in the latter by polyacrylamide gel electrophoresis followed by passage through G25 Sephadex. Similar results were obtained with oligonucleotides from these two sources. The *G0S30* phosphorothioated antisense oligonucleotide (AGGGTAGTTGTCCAT) differed by 1 base from the nonphosphorothioated oligonucleotide used by Perez-Castillo *et al.* (164; the 5' base of the latter is a G). These antisense oligonucleotides are targeted to the 5' UTR of *G0S30* mRNA. One control oligonucleotide had a sequence generated by shuffling the antisense oligonucleotide sequence (GTGTGCATAGTTGAC; no change in base composition). Another control oligonucleotide had four substitutions relative to the antisense oligonucleotide (AGAGTTATTCTCCAT). It should be noted that none of our oligonucleotides have G quartets (166), whereas the antisense oligonucleotide used by Perez-Castillo's group did have a G quartet (164).

Cell Culture

Cell culture methods were carried out as described previously (Chapter 2 Materials and Methods, 167). A preincubation period of 24 hr preceded the time of addition of Con-A (defined as 0 hr). The latter was added to a final concentration of 200 μg/ml, which is optimum for activation, although inhibitory in long term culture (168). For this reason, in experiments involving [^{3}H]thymidine incorporation the final concentration was 30 μg/ml. All experiments were repeated at least three times with cells from different donors.

Preparation of RNA

Total RNA was prepared as previously outlined (Chapter 2 Materials and Methods).

Competitive polymerase chain reaction

The concentration of *G0S30* mRNA was determined using the reverse transcriptase-polymerase chain reaction procedure as previously described (Chapter 2 Materials and Methods; 105).

Immunoblotting

At various times, cells in Eppendorf microtubes were harvested by centrifugation, washed by briefly vortexing in cold saline, and directly lysed in RIPA buffer (1% Nonidet, 1% sodium deoxycholate, 0.1% $NaDodSO_4$, 50 mM Tris-Cl pH 7.5) containing protease inhibitors [5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM PMSF]. Equal amounts of protein were applied to an SDS -polyacrylamide (7.5%) gel under reducing conditions. Proteins were

electrophoretically transferred to a PVDF membrane (Immobilon, Millipore Corp., Bedford, MA), and G0S30 protein was detected using the ECL system (Amersham Corp., UK) with rabbit affinity-purified polyclonal antibody raised against a carboxyterminal peptide (14 amino acids) of mouse EGR1 (Santa Cruz Biotechnology, CA). Detection by the antibody of the human homolog (a major band of approx. 86 kD as determined relative to protein standards; 169) is prevented by co-incubation with the peptide. Inositol polyphosphate 5-phosphatase was also detected using the ECL system (Amersham Corp., UK) with rabbit affinity-purified polyclonal antibody raised against a recombinant peptide (556 amino acids) of inositol polyphosphate 5-phosphatase (generous gift from Dr. Chris Mitchell, Monash University, Australia). Detection by the antibody of the human homolog revealed a major band of approx. 75 kD as determined relative to protein standards.

Incorporation of radioactive nucleosides

In a typical experiment cells (1.5×10^6 /ml) were preincubated in 75 ml of culture medium and autologous serum for 24 hr in a 250 ml tissue culture flask. Nonadherent cells ($\sim 1.2 \times 10^6$ /ml) were then transferred to a new flask, and 0.5-ml volumes were pipetted into Eppendorf microtubes containing phosphorothioated oligonucleotide. At 1.5 hr later, Con-A was added (0 hr). The incorporation of [5-^{3}H]uridine (1 μCi/ml; 814 Gbq/mmole) added at the same time as Con-A was assessed after a further 4-24 hr of culture. Alternatively, culture was continued for a further 44 hr when [^{3}H]thymidine (1 μCi/ml) was added and cells were harvested 9 hr later. Cells were harvested by centrifugation at 1500 g for 4min. After washing pellets with cold NaCl (0.14 N), the pellets were extracted with $HClO_4$ at 0°C for

10 min in order to precipitate macromolecules (i.e. RNA) and then washed with ethanol. The residues were dissolved in 0.4 ml hyamine hydroxide and then washed into a vial with 10 ml of toluene scintillator containing 2, 5-diphenyloxazole (5 g/l). Values of c.p.m. were obtained using the LS7500 Liquid Scintillation System (Beckman) with a counting time of 10 min during which sufficient counts were accumulated for statistical purposes. For a given experimental point, between two and four cultures were employed, depending on the circumstance. Each point is accompanied by the standard error of the mean (SEM) rather than the standard deviation (SD) since SD can be calculated if desired from the SEM values, and the latter are more visually compact. The variation between duplicates (or triplet, tetraplet) cultures in a given experiment would reflect the cumulative effect of variation at the levels of pipetting, harvesting and counting. At a higher level, there would be variation in the individual human donors of cells and serum and variations in the cell preparation procedures. Thus, all experiments were repeated at least three times with cells from different donors.

RESULTS

No evidence that complementary oligonucleotide targets G0S30

We have previously shown by RNA blotting that *G0S30* mRNA levels are greatly increased within 2 hr of the addition of Con-A to freshly cultured blood mononuclear cells. In these cultures, there is a significant basal level of expression in control cultures without Con-A, which rapidly declines (37). Confirming the mRNA induction of *G0S30* noted in Chapter 2, figure 3-1A shows the response to Con-A of cultures preincubated for a day, using

a sensitive PCR assay. At this time, the level of expression in control cultures is very low. The mRNA concentration of another differentially expressed transcript, derived from the *G0S19* cytokine gene, rises more slowly (Fig. 3-1B).

There is a substantial level of G0S30 protein in control cultures preincubated for a day that remained constant over the 4 hr period examined (data not shown). The concentration increases rapidly in response to Con-A, and is maintained for a least 4 hr (Fig. 3-2A). Preincubation of cells for 1.5 hr with the human equivalent of the antisense oligonucleotide used by Perez-Castillo *et al.* (164) in a rodent system (differing only by 1 base) does not affect G0S30 protein levels. Absence of an effect is observed in control and Con-A-treated cultures both at a low "physiological" concentration of the phosphorothioated oligonucleotide (50 nM; Fig. 3-2B, upper) and at a higher concentration (2 μM; Fig. 3-2B, lower). This was found at several time points (0.5 hr, 1 hr, 2 hr, 4 hr; data shown only for the 2 hr time point). Under the same conditions, the oligonucleotide also does not affect *G0S30* mRNA concentrations, as assessed using the above PCR assay (Fig. 3-3). Similar lack of effects were obtained with the shuffled control oligonucleotide (Fig. 3-3). Thus, at least in this human system, there is no evidence that the complementary antisense oligonucleotides targets *G0S30* at either the genomic or the mRNA level. This suggests that the oligonucleotide might inhibit lymphocyte proliferation by targeting some other gene, or by some other mechanism (170, 171).

Figure 3-1. **Effect of Con-A on levels of mRNA for (A) *G0S30/EGR1* and (B) the *G0S19* cytokine gene (human MIP1α) in cultured human blood mononuclear cells.** Figure illustrates one representative experiment. Filled symbols correspond to cultures with Con-A. Open symbols correspond to cultures without Con-A. Each point represents a single determination using reverse transcription and the polymerase chain reaction. Cells were preincubated for 24 hr prior to addition of Con-A at 0 min.

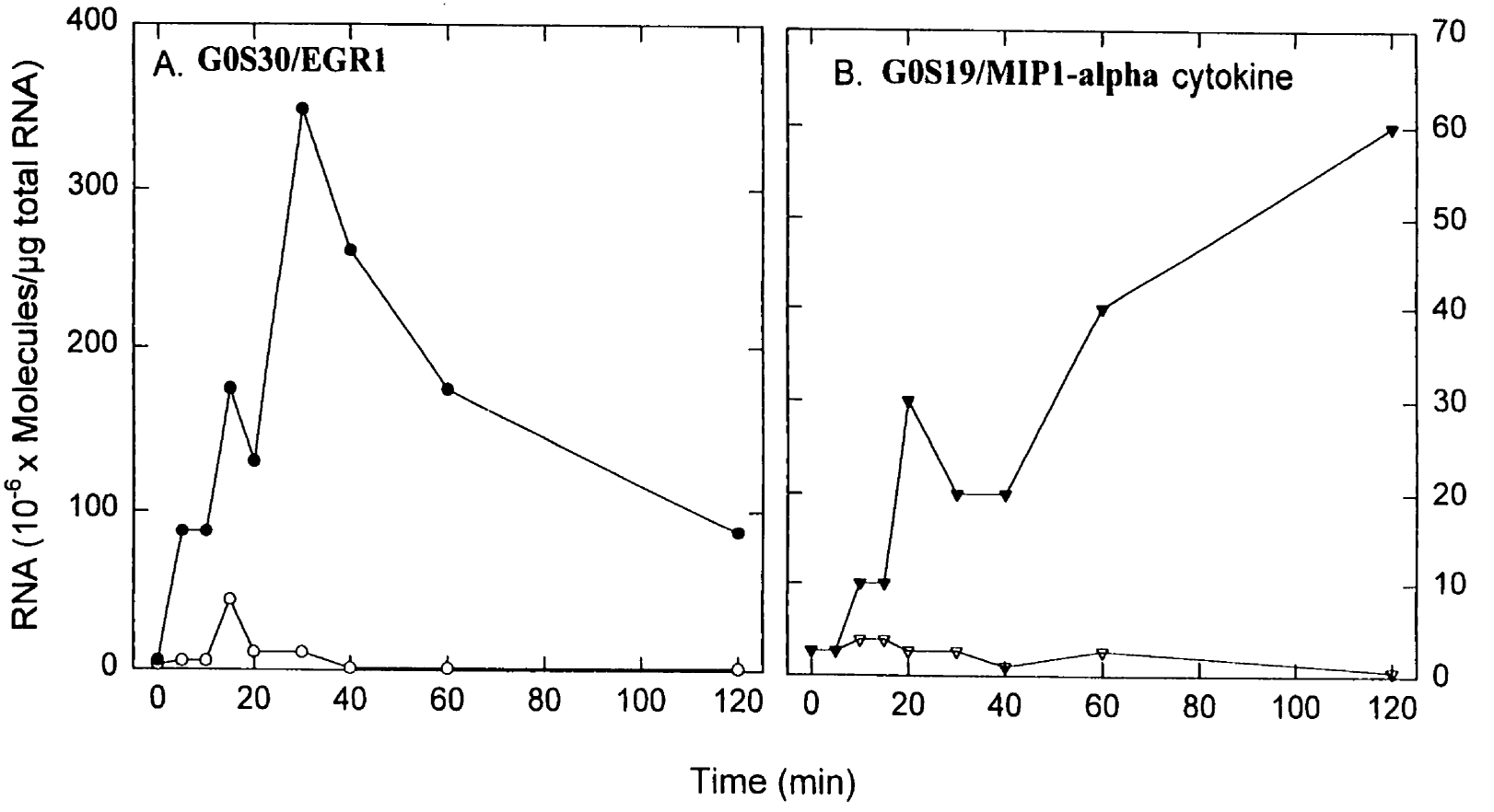

A. G0S30/EGR1
B. G0S19/MIP1-alpha cytokine
RNA (10-6 x Molecules/µg total RNA)
Time (min)
400
300
200
100
0
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120

Figure 3-2. **Absence of an effect of oligonucleotide complementary to *G0S30* on the concentration of G0S30 protein in cultures of Con-A-stimulated blood mononuclear cells.** Figure illustrates one representative experiment. Cells were preincubated for 24 hr prior to the addition of Con-A. (A) Immunoblot detection of G0S30 protein at various times after the addition of Con-A in the absence of added oligonucleotides. The faint band in the first lane is a 97-kD internal marker, which is recognized by secondary antibody in the ECL system. (B) In a separate experiment, the test and control ("shuffled") oligonucleotides were added at concentrations of either 50 nM (upper) or 2 μM (lower), 1.5 hr prior to the addition of Con-A. Cells were lysed for protein determination after 2 hr of further culture. Relative band intensities are indicated by numbers below the bands (densitometry scans). In addition to the major band (~86 kD), prolonged exposure of the film used for band detection revealed faint bands at ~129 kD and 64 kD. The intensities of these bands were also not affected by oligonucleotides.

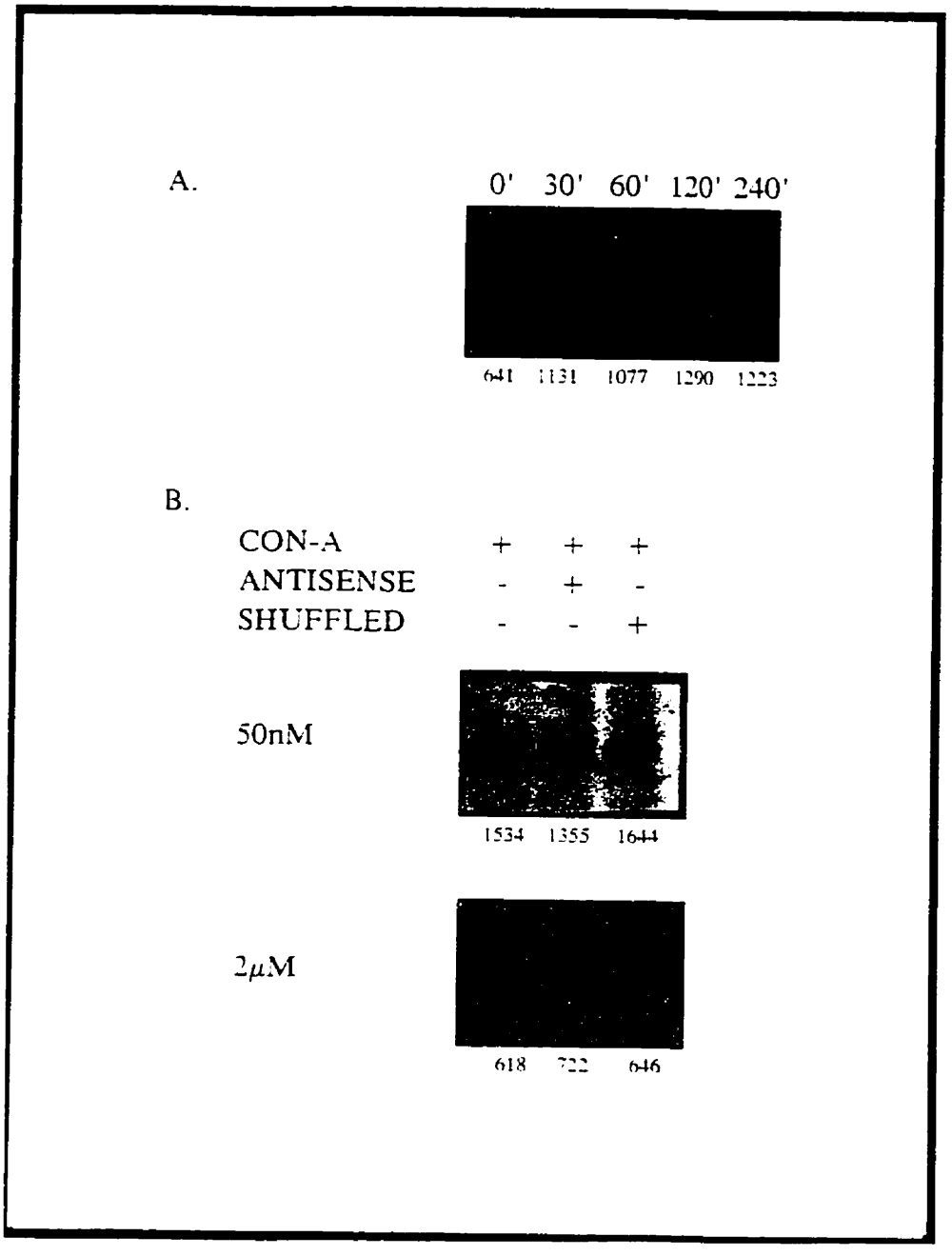
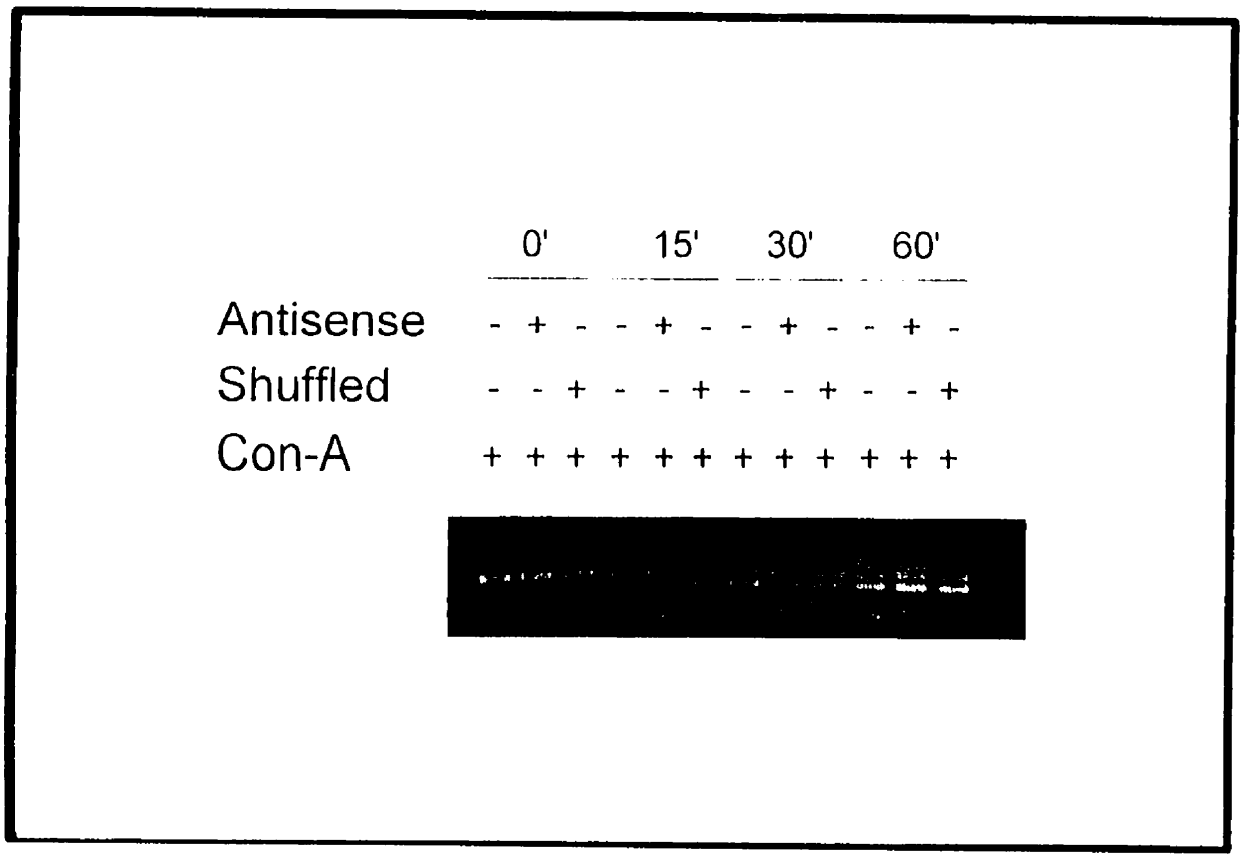

A.
0' 30' 60' 120' 240'
641 1131 1077 1290 1223
B.
CON-A + + +
ANTISENSE - + -
SHUFFLED - - +
50nM
1534 1355 1644
2μM
618 722 646

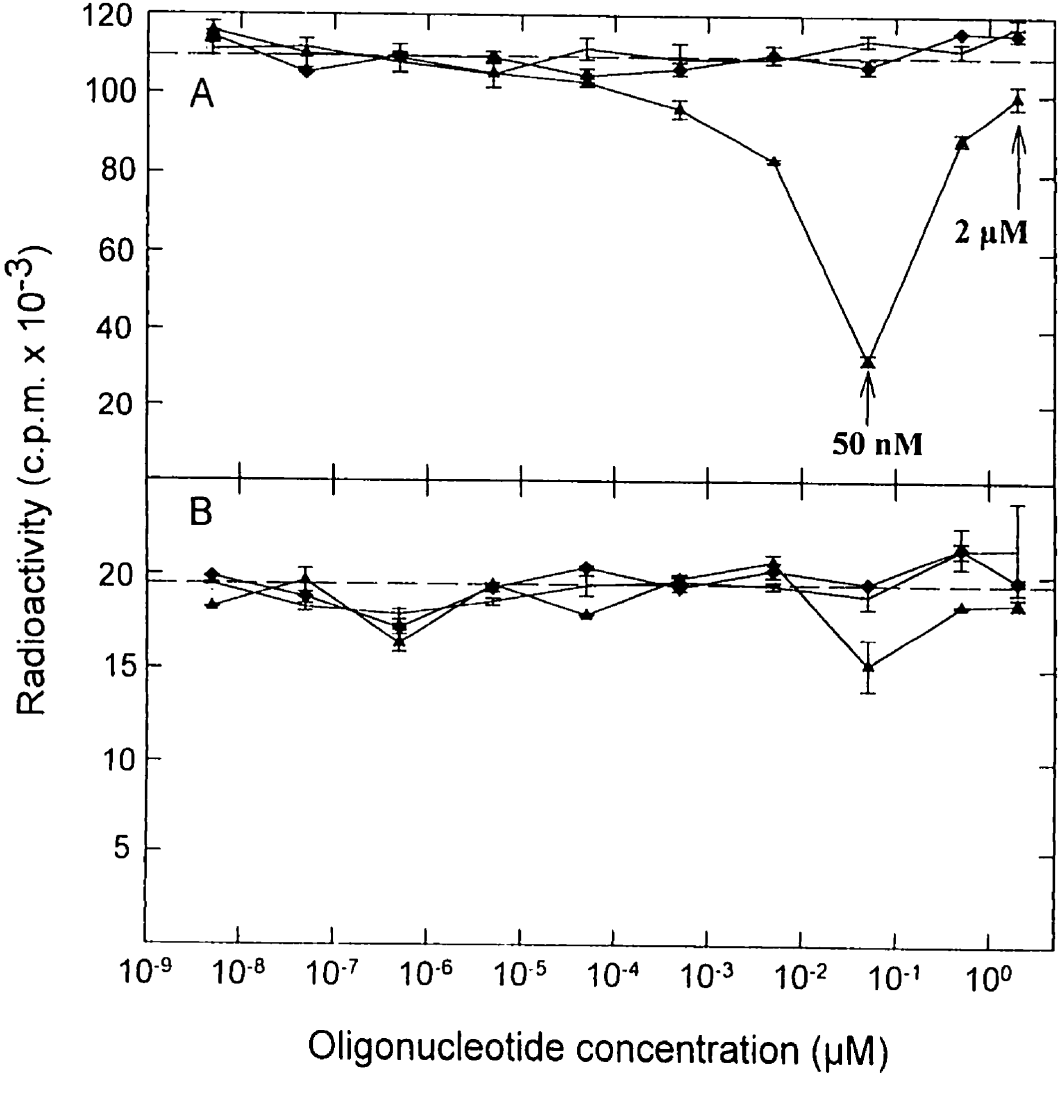
Figure 3-3. **Absence of an effect by *G0S30* -complementary antisense oligonucleotide and control oligonucleotide on the concentration of *G0S30* mRNA in cultures of Con-A-stimulated blood mononuclear cells.** Figure illustrates one representative experiment. Cells were preincubated for 24 hr prior to the addition of either *G0S30* antisense or control ("shuffled") oligonucleotides. After an additional 1.5 hr incubation, Con-A was added. After purification of RNA, samples were quantitated and reverse transcribed using a *G0S30*-specific primer. Resulting cDNA was analyzed by competitive PCR using *G0S30*-specific primers and a *G0S30* control cDNA. PCR products were separated on a 0.8% agarose gel, followed by ethidium bromide staining. Upper band indicates PCR product of *G0S30* control cDNA template whereas lower band indicates PCR products of reverse transcribed template.


0'
15'
30'
60'
Antisense
- + - - + - - + - - + -
Shuffled
- - + - - + - - + - - +
Con-A
+ + + + + + + + + + + +

Inhibition by "physiological" oligonucleotide concentrations

Con-A-induced lymphocyte proliferation can be influenced by targeting early events (e.g., the G_0/G_1 switch), or late events (e.g., the G_1/S phase transition). Because rat spleen cell proliferation was shown by Perez-Castillo and co-workers (164) to be inhibited by a labile nonphosphorothioated oligonucleotide added 2 hr prior to cell activation, the oligonucleotide may have targeted an early event. Thus, we examined whether our more stable phosphorothioated oligonucleotide would inhibit during the period of the G_0/G_1 switch and early G_1 progression as assessed by incorporation of [^{3}H]uridine into RNA (172). Figure 3-4A shows the result of preincubating cells for 1.5 hr with various concentrations of the test oligonucleotide complementary to *G0S30*, prior to adding Con-A and [^{3}H]uridine. The oligonucleotide was added at concentrations of 2 μM, 0.5 μM, and a series of 10-fold dilutions of the latter concentration. Acid-precipitable radioactivity was assessed after a further 22 hr of culture. The horizontal dashed line indicates the level of radioactivity in Con-A-treated cultures without added oligonucleotide. Most inhibition is achieved at a concentration of 50 nM, which is close to the "physiological" concentration of phosphorothioated oligonucleotide employed by Krieg and co-workers (163). Below this concentration, the inhibition progressively decreases, indicating that oligonucleotide concentration is limiting. The inhibition also decreased when the oligonucleotide concentration was *increased* above 50 nM, consistent with the endogenous defense system hypothesis. The specificity of the inhibition was examined using the two control oligonucleotides, neither of which were able to inhibit at the concentrations shown. Figure 3-4B shows that inhibition by 50 nM test oligonucleotide is also detectable when DNA

Figure 3-4. **Dose-response curves for oligonucleotide inhibition of (a) RNA synthesis or (b) DNA synthesis by cultured blood mononuclear cells activated by Con-A.** Figure illustrates one representative experiment. Cells (1.5×10^6/ml) were preincubated for 24 hr prior to the addition of Con-A. In (A), [^{3}H]uridine was added with Con-A (0 hr), and cells were harvested at 22 hr. In (B), [^{3}H]thymidine was added 44 hr after Con-A and cells were harvested 9 hr later. Closed triangles, the test *G0S30* antisense oligonucleotide; open diamonds, a control oligonucleotide with a sequence generated by shuffling the antisense oligonucleotide sequence; closed diamonds, a control oligonucleotide with four substitutions relative to the antisense oligonucleotide. The horizontal dashed lines indicate the level of radioactivity incorporated in the absence of added oligonucleotides (mean of quadruplet cultures). Each point is the mean of duplicate cultures (± SEM). In the case of points shown without error bars, the limits fell within the area covered by the point.


120
100
80
60
40
20
A
2 µM
50 nM
B
20
15
10
5
Radioactivity (c.p.m. x 10-3)
10-9
10-8
10-7
10-6
10-5
10-4
10-3
10-2
10-1
100
Oligonucleotide concentration (µM)

synthesis is monitored using [^{3}H]thymidine incorporation after 44 hr of culture. but the inhibition is much less than when RNA synthesis is monitored at 22 hr of culture.

Narrow concentration range for maximum inhibition

Figure 3-5 shows an experiment similar to that shown in Fig. 3-4A. except that a narrow range of oligonucleotide concentrations was used. and the culture period was 6 hr. A 50 nM concentration of the test oligonucleotide reduces radioactivity to a level similar to that of control cultures without Con-A (lower horizontal dashed line). Small changes in concentration above or below this value greatly decrease this inhibition. Over this narrow range of concentrations, the control oligonucleotides were sometimes inhibitory. These inhibitions differed from the inhibition by 50 nM test oligonucleotide in always being at concentrations higher than 50 nM. By this criterion, the inhibition by the test oligonucleotide at 50 nM is specific. In some experiments. as in Fig. 3-5, high concentrations of all three oligonucleotides were inhibitory, indicating nonspecific affects.

High inhibition in early hours of culture

The degree of inhibition at different times of culture was examined more closely. Figure 3-6A shows the effects of 50 nM concentrations of the test and control oligonucleotides. These were pre-added at -1.5 hr, and RNA labelling with [^{3}H]uridine added at the same time as Con-A (0 hr) was assessed at various time points. In cultures without Con-A, small but inconsistent effects of the oligonucleotides are observed. The specific inhibition is observed at 4 hr after inhibition of Con-A, and can approach 100%. This is best

Figure 3-5. **Inhibition of RNA synthesis by the test oligonucleotide examined over a narrow range of concentrations.** Figure illustrates one representative experiment. Cells (1.1×10^6/ml) were preincubated for 24 hr prior to the addition of Con-A. Details are as in Fig. 3-4 except that the culture period with Con-A and [^{3}H]uridine was 6 hr. The upper horizontal dashed line indicates the level of radioactivity in Con-A-treated cultures without added oligonucleotide. The lower dashed line indicates the levels of radioactivity in cultures without Con-A or oligonucleotide.

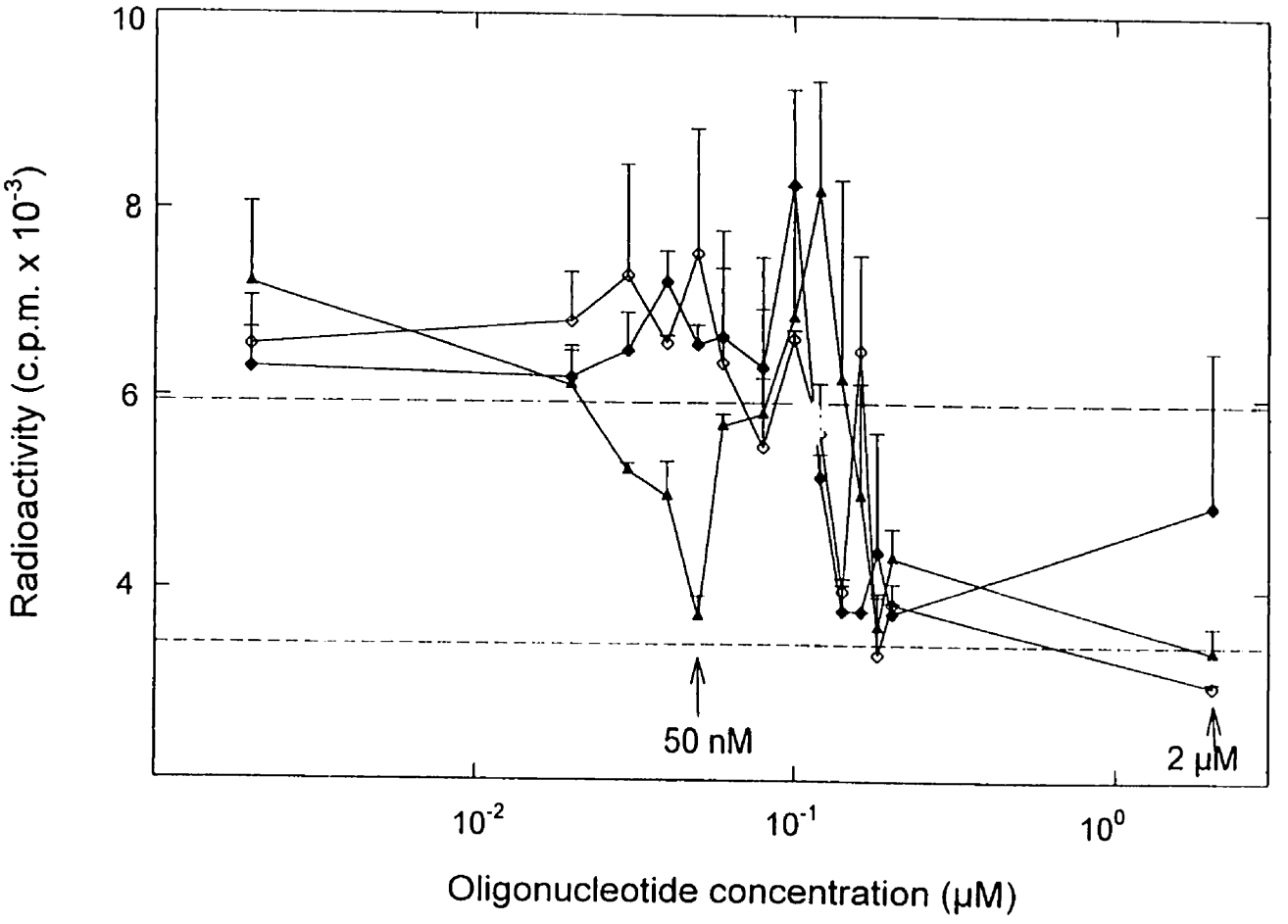
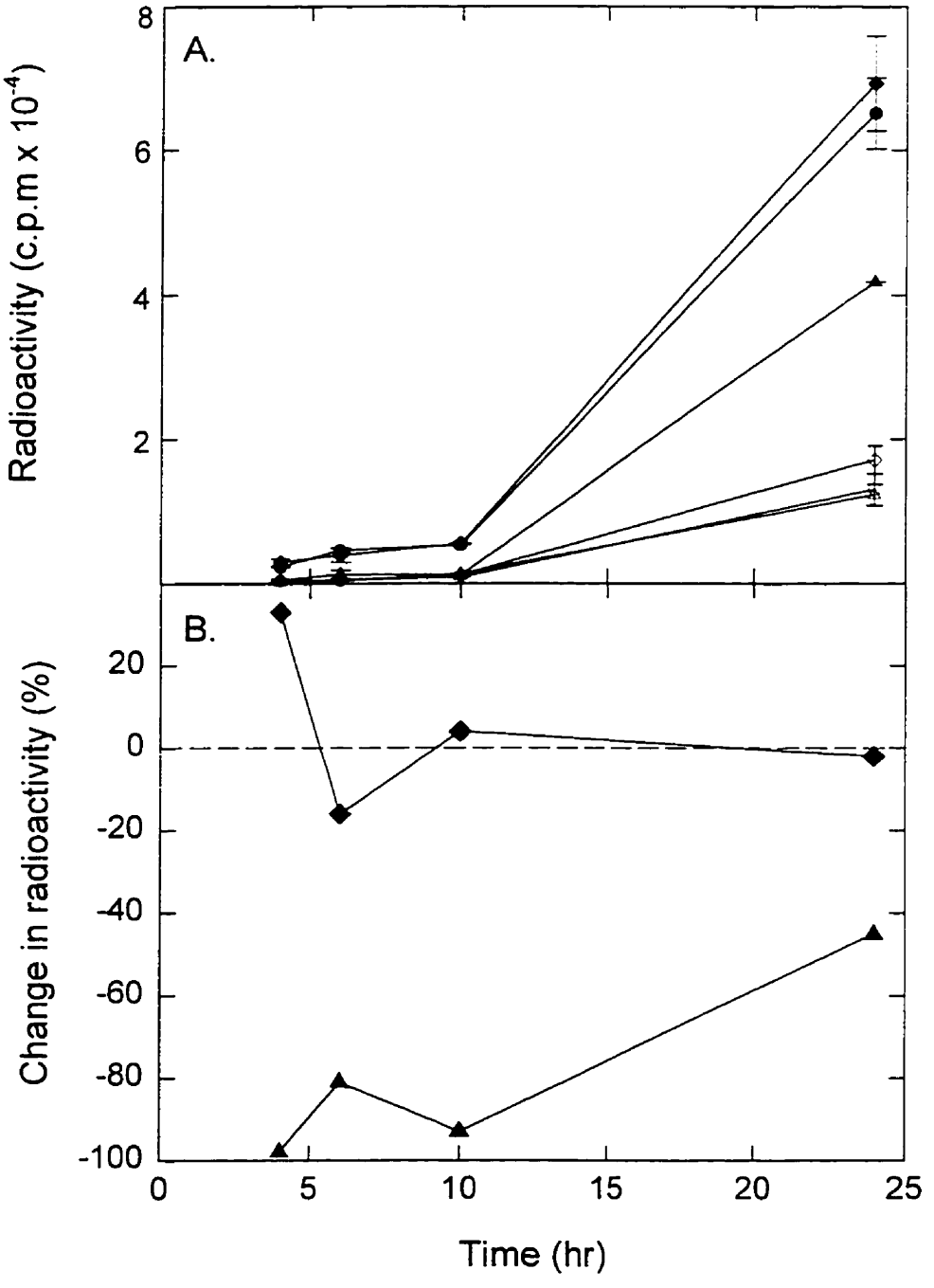

10
8
6
4
Radioactivity (c.p.m. x 10^{-3})
50 nM
2 μM
10^{-2}
10^{-1}
10^{0}
Oligonucleotide concentration (μM)

Figure 3-6. **Time course of effects of no added oligonucleotide (circles), 50 nM test oligonucleotide (triangles), and 50 nM control "shuffled" oligonucleotide (diamonds) on RNA synthesis in Con-A-treated cultures (closed symbols) and cultures without Con-A (open symbols).** Figure illustrates one representative experiment. Cells (1.9×10^6/ml) were preincubated for 24 hr and oligonucleotides were added 1.5 hr prior to the addition of Con-A (200 μg/ml) and [^{3}H]uridine. In (A), acid-precipitable radioactivity is plotted directly. Values for the 4-hr time points represent the mean of triplicate cultures. Later time points represent the mean of duplicate cultures. In (B), radioactivity in Con-A-treated cultures containing oligonucleotides is expressed relative to: (i) that of cultures containing Con-A and no added oligonucleotides (upper horizontal dashed line, corresponding to zero inhibition), and (ii) the baseline of labeling in cultures containing no Con-A and no added oligonucleotides (corresponding to 100% inhibition). These frames of reference were each the means of quadruplicate cultures. Other details are as in Fig. 3-4.


A.
B.
Radioactivity (c.p.m x 10^{-4})
Change in radioactivity (%)
Time (hr)
8
6
4
2
20
0
-20
-40
-60
-80
-100
0
5
10
15
20
25

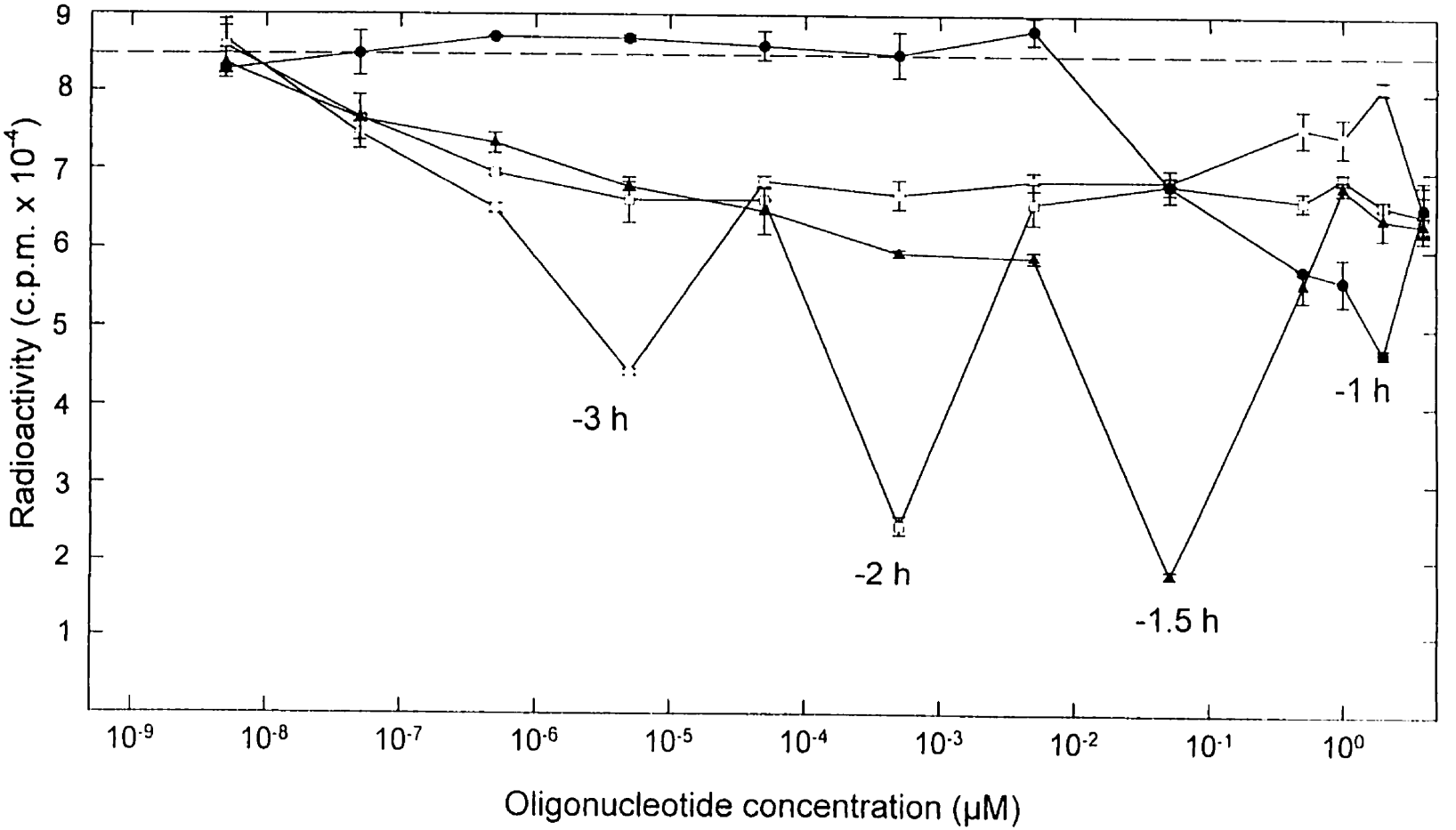
shown in Fig. 3-6B. in which percentage inhibitions relative to Con-A-treated cultures without added oligonucleotide. are plotted. The degree of inhibition becomes progressively less throughout the culture period. but is still substantial after 24 hr. These results indicate that the test oligonucleotide is able to inhibit some process (es) without which the G_0/G_1 switch and/or early G_1 progression cannot occur. The nature of the partial escape from inhibition throughout the G_1 phase is uncertain. but could reflect oligonucleotide degradation.

Inhibition depends on preincubation time

Figure 3-7 shows another experiment similar to that shown in Fig. 3-4a. In this case. the test oligonucleotide was added at either 3, 2, 1.5, or 1 hr prior to the addition of Con-A. Progress through the G_1 phase was monitored as [^{3}H]uridine incorporation into RNA over 22 hr of culture. Both the extent of the maximum inhibition and the oligonucleotide concentration required vary with preincubation time. Maximum inhibition is observed when cells are preincubated with 50 nM oligonucleotide for 1.5 hr. Moreover. maximum inhibitions are achieved with lower oligonucleotide concentrations when the preincubation time is extended, but the extent of the inhibition is less. With a shorter preincubation time (1 hr), the concentration for maximum inhibition is higher (2 μM) and the extent of inhibition is less. No inhibition is achieved with oligonucleotides added at the same time as Con-A (data not shown). Because 1.5 hr is ample time for oligonucleotide entry into cells (173), these observations are consistent with early inhibition of some intracellular process.

The oligonucleotide requirements for inhibition using the 3-hr and 2-hr preincubation periods were not examined over a narrow range of concentrations as in Fig. 3-5. Thus, it is

Figure 3-7. **Dose-response curves for test *G0S30* antisense oligonucleotide preincubated with cultured blood mononuclear cells for varying periods (open circles, 3 hr; squares, 2 hr; triangles, 1.5 hr; closed circles, 1 hr) prior to adding Con-A and [^{3}H]uridine (0 hr).** Figure illustrates one representative experiment. Cells (1.3×10^6/ml) were harvested at 22 hr for the determination for acid-precipitable radioactivity. The horizontal dashed line indicates the level of radioactivity in cultures with Con-A but no added oligonucleotide. Each point is the mean of duplicate cultures. Other details are as in Fig. 3-4.


Radioactivity (c.p.m. x 10^{-4})
9
8
7
6
5
4
3
2
1
-3 h
-2 h
-1.5 h
-1 h
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}
Oligonucleotide concentration (μM)

possible that greater inhibitions are possible at certain critical concentrations. The results in Fig. 3-7 show that a preincubation period of 1.5 hr is necessary to maximize inhibition by the test oligonucleotide at 50 nM. This concentration is much less inhibitory when the preincubation period is extended, consistent with the postulated endogenous defense system having been tripped by the time the target had appeared in a vulnerable form (e.g. increased synthesis of a critical mRNA).

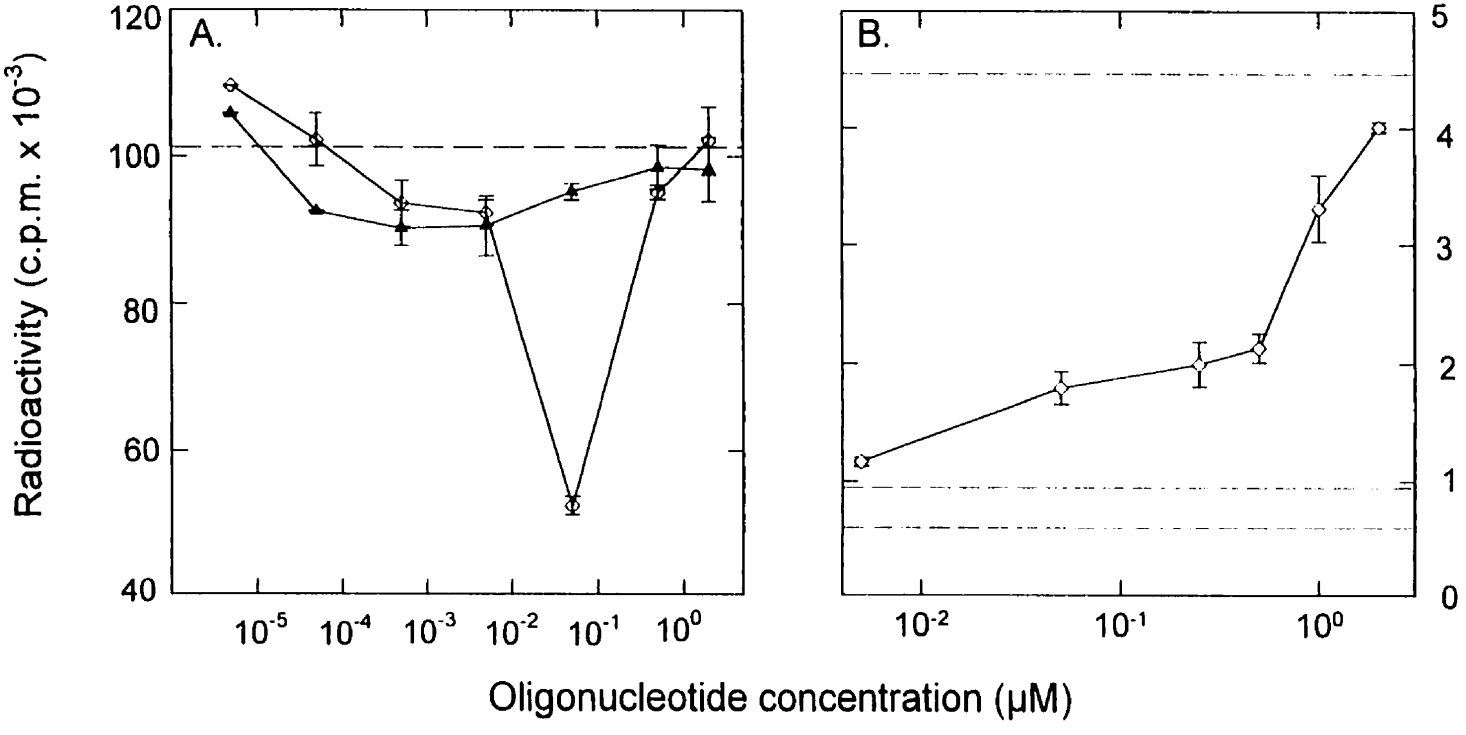
Oligonucleotides nonspecifically "trip" an endogenous defense system?

Above a critical oligonucleotide concentration the inhibition is greatly decreased and, in some experiments, is entirely eliminated (Figs. 3-4, 3-5, and 3-7). Figure 3-8A shows that when a fixed concentration (2 μM) of the control "shuffled" oligonucleotide is added at the same time as various concentrations of the test oligonucleotide (1.5 hr before adding Con-A) the inhibition by the latter at 50 nM is eliminated. Figure 3-8B shows the dose-dependence of this effect. In this case, the concentration of test oligonucleotide is kept constant (50 nM) and the control oligonucleotide concentration is varied. Similar results were obtained using the control oligonucleotide with 4-base differences (data not shown). Thus, whereas the inhibition at 50 nM by the test oligonucleotide is specific, the loss of inhibition at higher oligonucleotide concentrations is not oligonucleotide specific.

DISCUSSION

Blood mononuclear cells include a high proportion of T lymphocytes arrested in the G_0 phase of the cell cycle. Following activation by Con-A, cultured T lymphocytes enter the

Figure 3-8. **Prevention by critical concentrations of control oligonucleotide of inhibition by the test oligonucleotide.** Figure illustrates one representative experiment. In (A), cells (1.9×10^6/ml) were preincubated for 1.5 hr with varying concentrations of the test oligonucleotide and either no other oligonucleotide (triangles), or the control "shuffled" oligonucleotide (diamonds; 2 μM). [^{3}H]uridine and Con-A were added at 0 hr, and cells were harvested at 22 hr for determination of acid-precipitable radioactivity. Each point is the mean of duplicate cultures. The horizontal dashed line refers to the radioactivity in Con-A-treated cultures without any added oligonucleotide. In (B), cells were preincubated with varying concentrations of the control oligonucleotide and a fixed concentration (50 nM) of the test oligonucleotide. Acid-precipitable radioactivity was determined after 6 hr of culture with Con-A and [^{3}H]uridine. Each point is the mean of triplicate cultures. The three horizontal dashed lines represent, from top to bottom, radioactivities (mean of quadruplicate cultures) (i) with Con-A alone, (ii) with Con-A and test antisense oligonucleotide, and (iii) with no Con-A or oligonucleotide.


A.
B.
120
100
80
60
40
5
4
3
2
1
0
Radioactivity (c.p.m. x 10-3)
10-5
10-4
10-3
10-2
10-1
100
10-2
10-1
100
Oligonucleotide concentration (μM)

G_1 phase and enlarge. They then enter S phase and divide (162). *G0S30* is one of a set of human putative G_0/G_1 switch regulatory genes (*G0S* genes) identified in our laboratory (9). The mRNA is differentially expressed in blood lymphocytes shortly after addition of Con-A or cycloheximide (6, 37). The sequence is highly conserved between *G0S30* and the subsequently identified rodent homologs (*EGR1, NGFI-A, Zif268, KROX24, TIS8*; 41); these are differentially expressed shortly after adding growth factors to G_0-arrested cell lines. Homologs are also found in lower vertebrates (174), and in yeast (175). The protein product is a zinc-finger transcriptional regulator that binds a specific sequence. It has been presumed that regulation of target genes containing this sequence is required for entry into, and/or early progression through, the G_1 phase of the cell cycle (41); this process may also activate latent intracellular pathogens (176, 177).

To obtain evidence that *G0S30* expression was important for lymphocyte activation, Perez-Castillo and co-workers (164) preincubated rat spleen lymphocytes for 2 hr with an unmodified (nonphosphorothioated) oligonucleotide complementary to the 5' noncoding region of the mRNA and then added Con-A. At 2 μM oligonucleotide concentrations, they obtained inhibition of [^{3}H]thymidine incorporation after 2 days of culture, and concluded that there was "a direct relationship between *NGFI-A [G0S30]* gene expression and cell proliferation." Regarding Con-A as an antigen analog (178), the work was subsequently cited as "establishing that an activation response to antigen receptor-generated signals requires *EGR1 [G0S30]* expression" (179). However, without measuring levels of target mRNA or proteins to evaluate specificity, it is difficult to exclude the possibility that an observed effect is due to actions other than inhibition of the intended target RNA (152). Although Perez-

Castillo and co-workers noted that the oligonucleotide did not affect levels of *NGFI-B* mRNA, they did not report the presence or absence of an effect on the levels of *G0S30/NGFI-A* mRNA (180).

Furthermore, there is a paradox mentioned in the Introduction to this paper. Lee and co-workers (165) noted that their observation of an intact proliferative response to Con-A of lymphocytes from mice in which *G0S30* had been deleted, was "in direct contrast" to the results of Perez-Castillo and co-workers (164), who used rat spleen cell cultures. If results from different species are comparable, then our results with human blood mononuclear cells would appear to resolve the paradox. Low concentrations of the test oligonucleotide seem capable of profoundly and specifically inhibiting lymphocyte activation (Figs. 3-4 - 3-7), but there is no evidence that this is achieved by decreasing the concentrations of *G0S30* mRNA (Fig. 3-2) or protein (Fig. 3-3). Thus, the oligonucleotide is probably acting by some other mechanism. In view of the very low concentration required to achieve inhibition, the mechanism is unlikely to involve direct interaction with our "growth factor" Con-A (170).

The target(s) of the test oligonucleotide remains to be determined. The oligonucleotide is maximally effective when added to cells 1.5 hr prior to the addition of Con-A, and is ineffective when added at the same time as Con-A. This is consistent with a critical oligonucleotide-sensitive event occurring shortly after the addition of Con-A. The 1.5 hr-preincubation would be needed for the oligonucleotide to accumulate to a sufficient concentration within cells. Although a GenBank search revealed inositol polyphosphate 5-phosphatase mRNA as a possible target (only one mismatch), immunoblot analysis showed that inositol polyphosphate 5-phosphatase protein levels were unaffected by the presence of

the test and control oligonucleotides (data not shown). However, it is possible that the target is common to an oligonucleotide subset, or to oligonucleotides in general (170, 171). In this circumstance, the test oligonucleotide might just happen to have properties allowing it to enter cells more readily than control oligonucleotides; thus, it might exert an oligonucleotide-dependent, non-sequence-specific, intracellular inhibitory effect at lower concentrations than the control oligonucleotides. In keeping with this, we were sometimes able to detect inhibitory effects of the control oligonucleotides at higher concentrations than those observed with the test oligonucleotide, particularly in the early hours of culture (Fig. 3-5).

On the other hand, it should be noted that both the test and control oligonucleotides could have been acting through "specific" antisense mechanisms. An unknown number of mRNA species may be important for the G_0/G_1 switch, and each of these could have potential sites for interactions with many oligonucleotides. A weak affinity for one of these oligonucleotides would be compensated for if a sufficient concentration of the oligonucleotide were present, but that concentration should not be so great that it would "trip" cellular defense mechanisms. Thus, "specificity" must usually be considered in a concentration context. By this criterion, the test oligonucleotide used in our work is highly specific and is likely to be inhibiting the expression of a particular gene. It is unlikely that the inhibition is due to a contaminant, which would be expected to be present both in test and control oligonucleotide samples. Furthermore, similar results were obtained with oligonucleotides from different suppliers who used different purification methods. It would be a very strange contaminant, indeed, that exerted less inhibition when oligonucleotide concentration is increased above 50 nM.

Using Con-A-stimulated rat lymphoid cells, Perez-Castillo *et al.* (164) achieved inhibition of proliferation with a nonphosphorothioated oligonucleotide at 2 μM. When confronted with relatively poor inhibition by oligonucleotides at this concentration, as in the present work (Fig. 3-4), most investigators have responded by increasing the oligonucleotide concentration. Thus, most studies of the uptake, fate, and action of oligonucleotides have been carried out using concentrations in the 2-80 μM range. Indeed, the first oligonucleotide reported to inhibit putative *G0S30/EGR1*-dependent effects in cultured human cells was "phosphorothioate-capped" and required at a concentration of 80 μM (47).

Using Con-A-activated human blood mononuclear cells, we have here presented evidence that inhibition of lymphocyte activation can be increased by lowering the concentration of oligonucleotide to the "physiological" concentration of 50 nM. Above this concentration, inhibition is less. The decrease in inhibition at concentrations above 50 nM can be induced by oligonucleotides with different sequences from the oligonucleotide complementary to *G0S30* (Fig. 3-8). This supports the hypothesis that the decrease reflects the nonspecific induction of an endogenous system that impedes the uptake of, sequesters, and/or inactivates foreign oligonucleotides. Our results raise the possibility that administration of antisense oligonucleotides at concentrations below that which would "trip" endogenous defense mechanisms might permit inhibition of specific genes *in vivo* (the "stealth" approach). This would include the genes of pathogens.

At 50 μM, exogenous oligonucleotides localize in granular cytoplasmic compartments (98). In light of the present work, it is possible that these high concentrations might have triggered an endogenous defense system, which might have trapped and

channelled the oligonucleotides to that particular cytosolic compartment for degradation. Where oligonucleotides administered at a 50 nM concentration would locate is not known. There is evidence that uptake processes depend critically on concentration. At low oligonucleotide concentrations, uptake would be by a saturable, receptor-mediated, process, with a high proportion of added oligonucleotide rapidly entering cells (173). Fluid-phase endocytosis would predominate at concentrations > 1 μM, and a smaller proportion of the added oligonucleotide would rapidly enter cells (173).

Oligonucleotides required *in vitro* at concentrations in the μM range, would require administration in mg quantities to achieve similar concentrations in extracellular fluids *in vivo* (181). Apart from the expense, there could be unpredictable side effects, perhaps due to the targeting of nucleic acid-binding proteins. Much less administered oligonucleotide would be needed to achieve the low concentrations that are shown to be effective in the present work. Thus, the cost would be less and there should be less chance of side effects. The present work demonstrates that such low concentrations could be effective, but it is not known whether this is some special characteristic of the particular oligonucleotide or gene under study. Thus, it will be important to evaluate other oligonucleotides and other genes at these low concentrations. Furthermore, Fig. 3-7 reveals that a concentration which is inhibitory (50 nM) when oligonucleotide is added 1.5 hr prior to the addition of Con-A, is much less inhibitory when added at earlier times. The putative endogenous defense system does appear eventually to be triggered by the 50 nM concentration. Thus, to be effective in some *in vivo* situation, a dose administered to achieve a 50 nM extracellular concentration might have only a small time "window" in which to act.

CHAPTER 4

Base Composition-Determined Stem-Loop Potential as a Possible Predictor of the Effectiveness of Antisense Oligonucleotides Targeted to G_0/G_1 Switch Gene *G0S3/FOSB*

ABSTRACT

Inhibiting gene expression by specific antisense oligonucleotides offers a powerful means to assess the critical roles that genes play (reviewed in 73, 74). However, the factors which determine why a particular antisense oligonucleotide is effective are not clearly understood. A bioinformatic analysis was carried out on several target mRNAs different regions of which had previously been arbitrarily targeted by several antisense oligonucleotides and the relative efficacy of protein inhibition by these oligonucleotides had been assessed (82, 83, 182-189). There was no consistent trend noted in the analysis of studies carried out in cultured cells. The only consistent finding was a negative linear relationship noted when comparing the extent of inhibition and FORS-M values in studies carried out in cell-free extracts. This finding indicates that more inhibition is noted with oligonucleotides targeted to regions of low base composition-determined stem-loop potential.

Using base composition-determined stem-loop potential as a guide, antisense oligonucleotides targeted to regions of G_0/G_1 switch gene *G0S3/FOSB* mRNA with high (ASCH) and low (ASCL) base composition-determined stem-loop potential were designed. The effects of these oligonucleotides on levels of (i) G0S3/FOSB protein, (ii) *G0S3/FOSB* mRNA and (iii) [^{3}H]uridine incorporation, were assessed.

At all concentrations examined, there was found to be no significant effect by

antisense and control (shuffled) oligonucleotides on G0S3/FOSB protein levels in Con-A-stimulated cultures relative to cultures treated with Con-A alone. However, at the very low oligonucleotide concentration of 0.5 nM, there was a significantly lower G0S3/FOSB protein level in Con-A-stimulated cultures pretreated with ASCH oligonucleotide relative to those pretreated with ASCL oligonucleotide.

G0S3/FOSB mRNA levels were not significantly altered by pretreatment of Con-A-induced cultures with any oligonucleotide. Moreover, there was no significant difference in Con-A-stimulated cultures pretreated with ASCH oligonucleotide relative to those pretreated with ASCL oligonucleotide.

The effects of oligonucleotides on RNA synthesis ([^{3}H]uridine incorporation) were assessed for cell cultures in the absence and presence of Con-A. Incorporations were found to be inhibited by *both* ASCH oligonucleotide and ASCL oligonucleotide in control cultures without Con-A. However, no inhibition was seen in cultures stimulated with Con-A. These results suggest that in resting lymphocytes, basal levels of *G0S3/FOSB* mRNA are so low that G0S3/FOSB-dependent RNA synthesis can be inhibited by both the ASCH oligonucleotide and ASCL oligonucleotide. However, upon Con-A induction *G0S3/FOSB* mRNA levels greatly increase and thus the achievable intracellular oligonucleotide concentrations are insufficient to inhibit. Since ASCH and ASCL oligonucleotides were equally effective in control cultures, computational analysis of RNA secondary structure does not appear helpful in predicting oligonucleotide effectiveness.

INTRODUCTION

The sequence of the putative G_0/G_1 switch gene, *G0S3*, shows high homology with that of murine *FOSB*, which encodes a component of the AP-1 transcriptional regulator. The DNA binding domain of AP-1 is found in the promoter regions of several genes including that of cytokine IL2, which is involved in G_1 progression. It is therefore believed that G0S3/FOSB plays an important role in lymphocyte activation and/or progression through the G_1 phase of the cell cycle. The use of antisense oligonucleotides targeted to *G0S3/FOSB* mRNA might enable us to inhibit the expression of this immediate-early gene and thus inhibit G0S3/FOSB-dependent downstream events.

Although antisense oligonucleotides have proven to be very effective in inhibiting the expression of many target genes (reviewed in 73, 74), selection of an effective oligonucleotide is arbitrary. This "hit-and-miss" procedure can be very time consuming and expensive. As noted in the previous chapter, targeting a particular *G0S* gene mRNA with a complementary antisense oligonucleotide does not ensure that expression of that target gene will be inhibited. Therefore, prior to commencing an antisense oligonucleotide inhibition study of *G0S3/FOSB*, we decided to undertake a computational analysis to identify potential predictors of effective antisense oligonucleotide targeting sites. Such predictors might increase our chances of effectively targeting and inhibiting the expression of our gene of interest.

In the search for such a predictor, a number of studies have been carried out using computer algorithms which have examined various thermodynamic indices and kinetic models (79-81, 190). Some of these studies have attempted to correlate antisense

oligonucleotide inhibitory efficacy with RNA secondary structure by assessing the folding of the natural sequence for a target mRNA (191). Others have tried to correlate the extent of antisense oligonucleotide inhibition with $\Delta G_{formation}$ of the antisense oligonucleotide:mRNA target sequence duplex. Some have proposed that mapping RNA-accessible sites with oligonucleotide libraries can potentially unveil the most effective antisense oligonucleotides (192). Others have attempted to design more effective antisense oligonucleotides by first assessing the genomic frequency of target sequences in order to decrease the nonspecific binding activity of the oligonucleotides. Although much research has been dedicated to identify predictors of effective antisense oligonucleotides, findings have been inconclusive.

There are numerous studies in which arbitrarily chosen antisense oligonucleotides are directed to various regions of target gene mRNAs (82, 83, 182-189). These studies also report the extent of protein inhibition following addition of these various oligonucleotides. This chapter begins with a bioinformatic analysis of target gene RNA secondary structure, using the results of such studies (82, 83, 182-189), to investigate a potential correlation between some aspect of stem-loop potential and effective antisense oligonucleotide target sites. Although some researchers have considered RNA secondary structure to be an important indicator of oligonucleotide accessibility to its target site, bioinformatic studies have been limited to the analysis of minimum free energy values associated with the folding of the natural sequence (79). We can dissect this overall stem-loop potential into two component values of (i) *base composition*-determined stem-loop potential and (ii) *base order*-determined stem-loop potential (193). This allows us to investigate possible correlations between these individual components and oligonucleotide-mediated inhibition.

Using our computational analysis as a guide, we designed candidate inhibitory oligonucleotides. We then attempted to inhibit the expression of *G0S03/FOSB*. In this manner, we not only assessed our ability to predict effective antisense oligonucleotides, but also investigated the potentially pivotal role G0S3/FOSB may play in lymphocyte activation. Moreover, this study enabled us to further test previous findings (Chapter 3) that relatively low concentrations of antisense oligonucleotides are more effective in inhibiting target genes than higher concentrations.

MATERIALS AND METHODS

FONS, FORS-M and FORS-D analysis of target mRNA sequences

The folding of the natural sequence (FONS), folding of randomized sequence-mean (FORS-M), and folding of randomized sequence-difference (FORS-D) minimum free energy values were determined as previously described (82, 83, 182-189) using the LRNA program of Zuker (194). Briefly, overlapping windows (200 nt) were selected at 25-nt intervals (ie. 1-200, 25-225, 50-250, 75-275, 100-300, etc.). Secondary structure energy minimization values, determined using the program LRNA, were obtained for each window in the natural sequence (FONS values). Each 200-nt sequence was then subjected to 10 independent randomizations, and minimum energy values were determined for each of the randomized sequences. The mean minimum energy value for each set of 10 randomized sequences was then determined (FORS-M) as well as the differences (FORS-D) between these FORS-M values and the corresponding FONS values. The FORS-M value provides a measure of base composition-determined stem-loop potential and the FORS-D value evaluates the base order-

determined stem-loop potential. The FONS value incorporates both of these components. This bioinformatic analysis was carried out for several target mRNAs for which different regions had previously been targeted with several antisense oligonucleotides and the efficacy of protein inhibition by these oligonucleotides had been assessed (82, 83, 182-189). There was essentially one criterion for selection of mRNA targets to be used in our bioinformatic analysis. This criterion was that the target mRNA had been the focus of a study in which a number of antisense oligonucleotides targeted to various regions of the mRNA were tested and the varying degrees of inhibition reported. Combining this information with our study of RNA secondary structure, we assessed any potential correlation between RNA secondary structure and the extent of oligonucleotide-mediated inhibition.

Phosphorothioated oligonucleotides

Oligonucleotides were obtained from Cortec DNA Service Laboratories, Queen's University. Purification of the oligonucleotides was by thin-layer chromatography. The sequence of the *G0S03/FOSB* phosphorothioated antisense oligonucleotide targeted to a region of high base composition-determined stem-loop potential was GCTGTAGCCACTCAT (ASCH). The phosphorothioated antisense oligonucleotide targeted to a region of low base composition-determined stem-loop potential was GACAAACGAAGAAGT (ASCL). Both antisense oligonucleotides targeted the coding regions of *G0S3/FOSB* mRNA. The control oligonucleotide had a sequence generated by shuffling the ASCH sequence (CCGAGACCTGATCTT; i.e. no change in base composition). Financial considerations precluded the possibility of further control

oligonucleotides.

Cell Culture

Cell culture method was carried out as previously described (Chapter 2 Materials and Methods).

Immunoblotting

At various times, cells in Eppendorf microtubes were harvested by centrifugation, washed by briefly vortexing in cold saline, and directly lysed in RIPA buffer (1% Nonidet, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-Cl pH 7.5) containing protease inhibitors [5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM PMSF]. Equal amounts of protein were applied to a SDS-polyacrylamide (7.5%) gel under reducing conditions. Proteins were electrophoretically transferred to a PVDF membrane (Immobilon, Millipore Corp., Bedford, MA), and G0S03 protein was detected using the ECL system (Amersham Corp., UK) with rabbit affinity-purified polyclonal antibody raised against a peptide corresponding to residues 102-117 (16 amino acids) of mouse FOSB (Santa Cruz Biotechnology, CA). Detection by the antibody of the human homolog (a major band of approx. 55 kD as determined relative to protein standards) is prevented by co-incubation with the peptide.

Preparation of RNA

Total RNA was prepared as described (Chapters 2, 3 Materials and Methods).

Reverse Transcription-Competitive polymerase chain reaction

RT-PCR was carried out as outlined (Chapters 2, 3 Materials and Methods).

Incorporation of radioactive nucleosides

[5-^{3}H]uridine incorporation was carried out as previously outlined (See Chapters 3 Materials and Methods).

Statistics

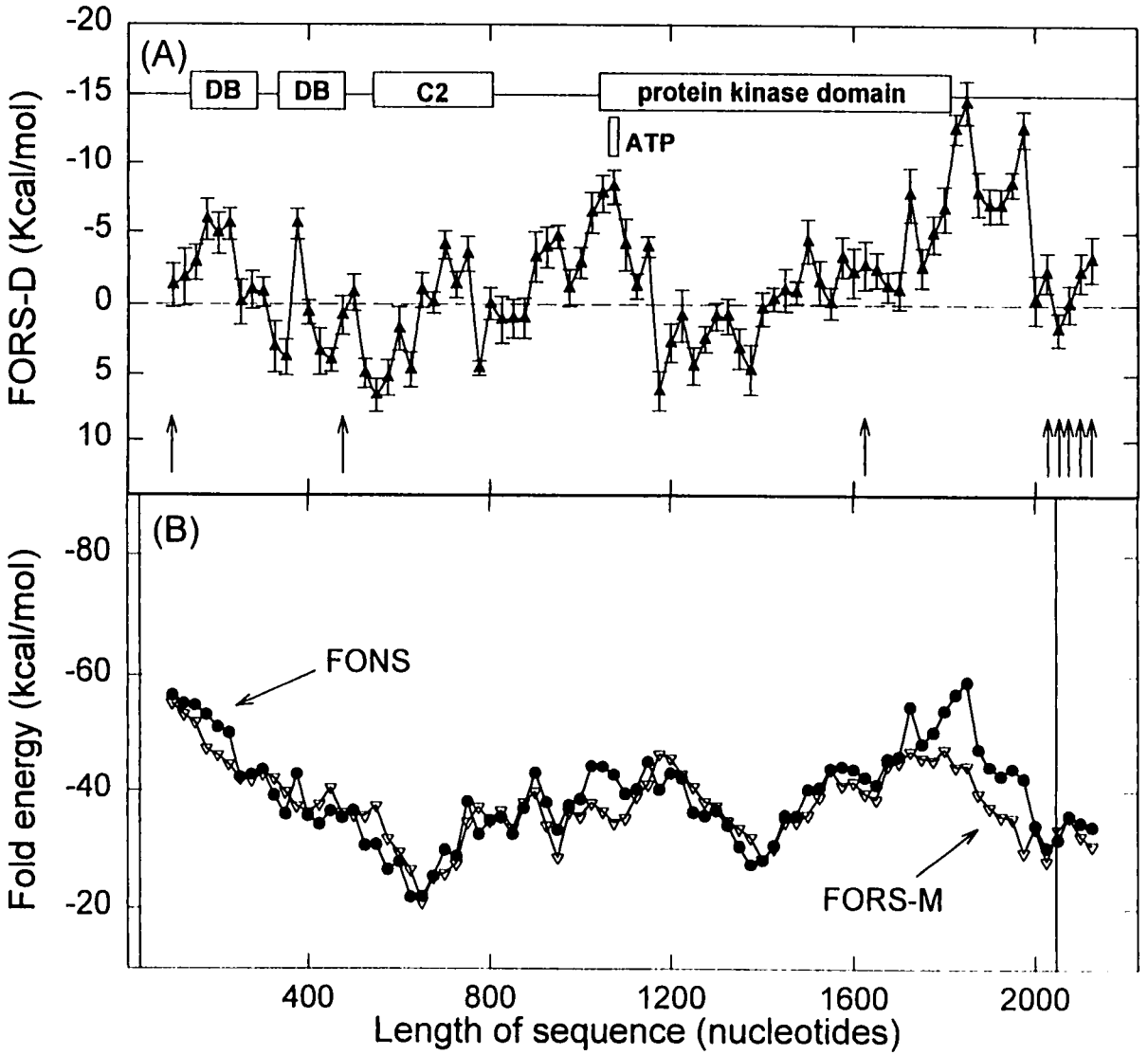
Statistical analyses were carried out as previously outlined (Chapter 2 Materials and Methods).

RESULTS

Is there a relationship between antisense oligonucleotide inhibitory efficacy and RNA secondary structure?

A bioinformatic analysis of RNA secondary structure was undertaken to investigate a possible correlation between the stem-loop potential of a region and effective inhibition by antisense oligonucleotides. For this analysis, various studies were employed (82, 83, 182-189) in which a number of antisense oligonucleotides, targeted to various regions of mRNA, had been tested for their ability to inhibit the expression of target genes. We initially assessed FONS, FORS-M, and FORS-D minimum free-energy values for overlapping windows of human *PKC-α* mRNA (Fig. 4-1). It is evident from Fig. 4-1B that the FORS-M and FONS values are very similar and deviate slightly throughout the mRNA sequence. This deviation

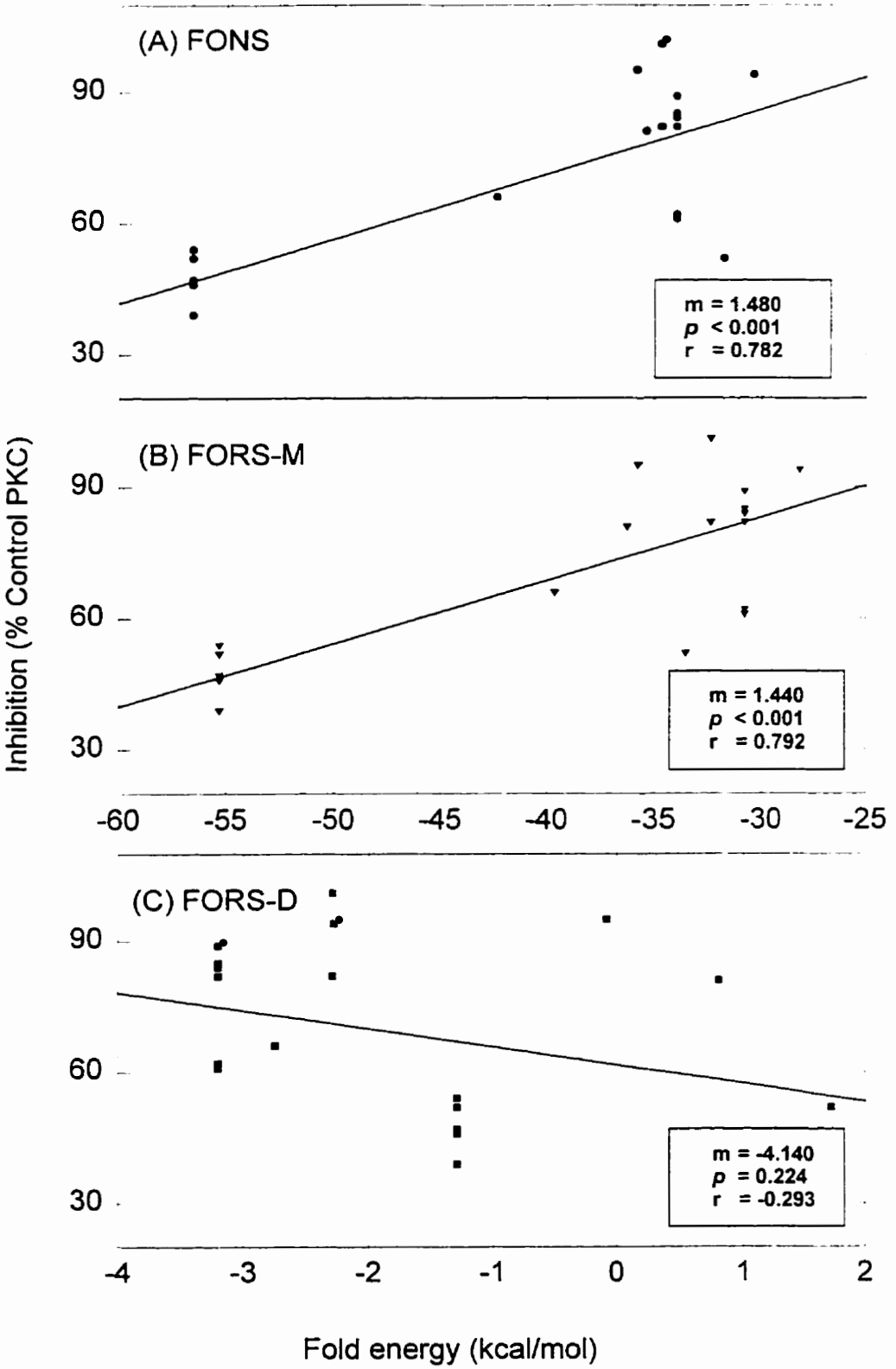
Figure 4-1. **Fold energy minimization values (FONS, FORS-M) and differences (FORS-D) for *PKC-α* mRNA.** Overlapping windows (200 nt) were selected at 25-nt intervals (ie. 1-200, 25-225, 50-250, 75-275, 100-300, etc.). Secondary structure energy minimization values, determined using the program LRNA, were obtained for each window in the natural sequence (FONS values). Each 200-nt sequence was then subjected to 10 independent randomizations, and minimum energy values were determined for each of the randomized sequences. The mean minimum energy value for a set of 10 randomized sequences (FORS-M) is plotted with the corresponding FONS value (B). The differences between FORS-M values and the corresponding FONS values are plotted (FORS-D ± standard error) (A). Each data point is at the middle of the 200-nt window. Arrows drawn in (A) indicate regions to which antisense oligonucleotides were targeted. Sometimes, several different oligonucleotides were targeted to the same region. Boxes drawn in (A) represent the following domains of the PKC-α: DB = diacylglycerol/phorbol ester binding domain, C2 = calcium phospholipid binding module, ATP = ATP binding domain. Vertical dashed lines in (B) represent boundaries of the coding region.


(A)
DB
DB
C2
protein kinase domain
ATP
FORS-D (Kcal/mol)
-20
-15
-10
-5
0
5
10
(B)
FONS
FORS-M
Fold energy (kcal/mol)
-80
-60
-40
-20
400
800
1200
1600
2000
Length of sequence (nucleotides)

gives rise to the FORS-D plot illustrated in Fig. 4-1A. In other words, the base composition-determined stem-loop potential (FORS-M value) closely follows the overall stem-loop potential (FONS value), and the differences between these values give rise to the base order-determined stem-loop potential (FORS-D value). Particular regions of the *PKC-α* nucleotide sequence in Fig. 4-1A (325 - 625 nt and 1175 -1400 nt) are noted as having low (highly positive FORS-D) base order-determined stem-loop potential. Such regions may be under evolutionary pressure to avoid forming stem-loop structures in order to encode a particular protein region (193, 195, 196). Not being able to accommodate *both* stem-loop structures and the coding of necessary protein domains, the former pressure may have been sacrificed for the latter.

Using these minimum energy values and the inhibition data from Dean *et al.* (182), relationships between antisense oligonucleotide-mediated inhibition and FONS (Fig. 4-2A), FORS-M (Fig. 4-2B), and FORS-D (Fig. 4-2C) values were assessed for PKC-α. Fig. 4-2A illustrates that more inhibition is noted when targeting mRNA regions with highly negative FONS values. Linear regression analysis gave a slope of this line (m=1.480) which was found to be significantly different from a slope of zero ($P<0.001$). This suggests that targeting antisense oligonucleotides to regions of mRNA with high potential to form stem-loop structures will result in the most effective inhibition. Fig. 4-2B reveals that base composition-determined stem-loop potential closely parallels the findings of Fig. 4-2A. Fig. 4-2C shows very different results. Here we notice that more inhibition is noted with highly positive FORS-D values (low base order-determined stem-loop potential). However, the slope (m=-4.140) of this curve does not differ significantly from a slope of zero ($P=0.224$).

Figure 4-2. **Relationship between minimum energy values and the extent of inhibition of PKC-α protein level.** Our minimum energy values (FONS, FORS-M) and differences (FORS-D) for *PKC-α* mRNA (see Fig. 4-1) were compared with the inhibition results of a previous study (122) in which several antisense oligonucleotides were targeted to various regions of *PKC-α* mRNA. Inhibition, expressed as percentage control (control being PKC-α protein levels in sample without antisense oligonucleotides added = 100%) is plotted with the corresponding FONS values (A), FORS-M values (B), and FORS-D values (C). Boxed numbers correspond to m = slope, *P* = observed significance level, and r = coefficient of correlation.


(A) FONS
90
60
30
m = 1.480
p < 0.001
r = 0.782
(B) FORS-M
90
60
30
m = 1.440
p < 0.001
r = 0.792
-60
-55
-50
-45
-40
-35
-30
-25
(C) FORS-D
90
60
30
m = -4.140
p = 0.224
r = -0.293
-4
-3
-2
-1
0
1
2
Inhibition (% Control PKC)
Fold energy (kcal/mol)

The results suggest that more inhibition will be achieved by targeting mRNA regions of high base composition-determined stem-loop potential. We note that our findings are highly dependent on a cluster of data points in the region of highly negative FORS-M values.

Other genes were analyzed similarly. This involved inhibition studies carried out both in cultured cells (Table 4-1) and in cell-free extracts (Table 4-2). When comparing inhibition and FONS values for cultured cells, there were 5 with negative slopes when comparing inhibition and FONS values (mean of 9 studies = -0.05 ± 1.09, $P = 0.374$; Table 4-1). Of the 4 studies that were found to have positive slopes, only *PKC-α* was found to have a slope that differs significantly from zero. Although a positive linear relationship was found between the extent of inhibition and FONS values for *PKC-α*, it was not found in our analysis of other target mRNAs. Of the 5 studies found to exhibit negative slopes, none were found to have slopes that differ significantly from zero.

FORS-M results were also not helpful. Of the 9 studies, 5 were found to have positive slopes (mean of 9 studies = 0.87 ± 0.92, $P = 0.361$; Table 4-1). Of these 5 studies, only 2 were found to have slopes that differ significantly from zero and fit a positive linear relationship between inhibition and FORS-M values. Of the 4 studies that exhibit negative slopes, none were found to have slopes that differ significantly from zero. Analysesof FORS-D values were also not helpful. Although 6 of the 9 studies analyzed were found to have negative slopes (mean of 9 studies = -0.48 ± 0.69, $P = 0.498$; Table 4-1), none of the slopes were found to differ significantly from zero. The 3 remaining studies demonstrated a positive slope that did not differ significantly from zero.

Our FONS analysis of the 5 inhibition studies carried out in cell-free extracts revealed

Table 4-1. Summary of Correlation Studies between Antisense Oligonucleotide-mediated Inhibition in cultured cells and FONS, FORS-M, and FORS-D Values

	Target	Oligo. Mod.	Oligo. length (nt)	[Oligo.] (μM)	FONS			FORS-M			FORS-D			Reference
					m	p	r	m	p	r	m	p	r	
	PKC-alpha	+	20	1.00	1.480	0.000	0.782	1.440	0.000	0.792	-4.140	0.224	-0.293	182
	VCAM1	+	20	0.05	-0.688	0.350	-0.260	-0.663	0.371	-0.249	0.183	0.921	0.028	186
	ELAM1	+	20	0.05	2.040	0.066	0.443	1.510	0.291	0.263	3.590	0.079	0.424	185
a	*ICAM1*	+	20	0.80	-1.320	0.152	-0.440	-1.090	0.257	-0.355	-1.640	0.480	-0.226	183
b	*ICAM1*	+	20	0.60	-0.599	0.545	-0.195	-0.447	0.660	-0.141	-1.010	0.677	-0.134	186
	MRP	+	20	0.50	-0.215	0.464	-0.205	0.002	0.933	0.032	-0.332	0.321	-0.276	188
	C-MYC	-	15	10.00	0.689	0.122	0.525	0.760	0.099	0.550	-0.070	0.960	0.000	187
	col1A1	+	18	0.20	-0.979	0.195	-0.423	-1.410	0.310	-0.338	0.613	0.459	0.249	83
	HBV	-	15	17.40	5.370	0.128	0.692	7.710	0.032	0.851	-1.510	0.873	-0.084	184
	Mean				-0.05 ± 1.09 (P=0.374)			0.87 ± 0.92 (P=0.361)			-0.48 ± 0.69 (P=0.498)			

Oligo. Mod.- antisense oligonucleotides were either phosphorothioated (+) or had no modification to the phosphodiester (-) backbone

Oligo. length - antisense oligonucleotides varied in length between 15 and 28 nucleotides as noted above

[Oligo.] - one antisense oligonucleotide concentration (μM) was used in each inhibition study shown above

Slope (m), observed significance level (P) and coefficient of correlation (r) values for relationships between Inhibition and FONS, FORS-M and FORS-D values are shown

a antisense oligonucleotides were added to human A549 lung carcinoma cells

b antisense oligonucleotides were added to human umbilical vein endothelial cells

Table 4-2. Summary of Correlation Studies between Antisense Oligonucleotide-mediated Inhibition in cell-free extracts and FONS, FORS-M, and FORS-D Values

	Target	Oligo. Mod.	Oligo. length (nt)	[Oligo.] (μM)	FONS			FORS-M			FORS-D			Reference
					m	p	r	m	p	r	m	p	r	
a	*beta-globin*	+	20	0.50	-7.270	0.081	-0.831	-3.650	0.025	-0.924	5.780	0.041	0.640	82
a	*beta-globin*	+	20	2.00	-3.360	0.124	-0.775	-1.650	0.075	-0.840	2.560	0.104	0.799	82
b	*beta-globin*	+	20	0.50	-1.550	0.475	-0.425	-0.058	0.955	-0.032	-0.690	0.676	-0.257	82
b	*beta-globin*	-	20	5.20	-2.400	0.499	-0.348	-1.530	0.358	-0.460	3.140	0.285	0.525	82
	HCV	+	28	10x excess	-2.140	0.160	-0.336	-2.210	0.387	-0.210	-3.840	0.137	-0.355	189
	Mean				-3.34 ± 1.02 (P=0.011)			-1.82 ± 0.58 (P=0.014)			1.39 ± 1.66 (P=0.428)			

Oligo. Mod.- antisense oligonucleotides were either phosphorothioated (+) or had no modification to the phosphodiester (-) backbone

Oligo. length - antisense oligonucleotides varied in length between 15 and 28 nucleotides as noted above

[Oligo.] - one antisense oligonucleotide concentration (μM) was used in each inhibition study shown above

Slope (m), observed significance level (P) and coefficient of correlation (r) values for relationships between Inhibition and FONS, FORS-M and FORS-D values are shown

a - antisense oligonucleotide inhibition studies were carried out in wheat germ extracts

b - antisense oligonucleotide inhibition studies were carried out in reticulocyte lysates

all having negative slopes (mean of 5 studies = -3.34 ± 1.02, $P = 0.011$; Table 4-2). However, 4 of these slopes were not found to differ significantly from zero, and only 1 was nearly significant. FORS-M analysis also revealed that all 5 studies produce negative slopes (mean of 5 studies = -1.82 ± 0.58, $P = 0.014$; Table 4-2). However, 3 of these slopes were not found to differ significantly from zero, 1 was significant and 1 was nearly significant. FORS-D analysis of the 5 studies revealed 3 with positive slopes (mean of 5 studies = 1.39 ± 1.66, $P = 0.428$; Table 4-2).

The only apparent consistent finding was the negative slope noted when comparing the extent of inhibition and FORS-M values in studies carried out in cell-free extracts (Table 4-2). This indicated more inhibition with oligonucleotides targeted to regions of low base composition-determined stem-loop potential. As this trend was noted in 5 studies that were carried out in only 3 independent laboratories that targeted 2 mRNAs, the study was not seen as complete. More studies are required in this category (cell extracts) to further test the consistency of this trend. No consistent trend was noted for the studies carried out in cultured cells (Table 4-1). Although our *G0S* gene inhibition studies are carried out using cultured cells, we decided to design candidate oligonucleotides targeted to *G0S3/FOSB* on the basis of base composition-determined stem-loop potential (FORS-M), the presumption being that oligonucleotides targeted to regions of low base composition-determined stem- loop potential would be more effective.

G0S3/FOSB antisense oligonucleotides targeted to regions of high and low base composition-determined stem-loop potential

An RNA secondary structure analysis was carried out using the mRNA sequence of a human putative G_0/G_1 switch regulatory gene *G0S3/FOSB* (Fig. 4-3A, 4-3B). From these profiles, we designed an antisense oligonucleotide (ASCH) targeted to a region of *G0S3/FOSB* mRNA with high base composition-determined stem-loop potential and an antisense oligonucleotide (ASCL) targeted to a region of low base composition-determined stem-loop potential (Fig. 4-3C). The difference between the two regions was 34 kcal/mol. These regions differed much less in base order-determined stem-loop potential (2 kcal/mol). A control oligonucleotide (SH; created by shuffling the sequence of ASCH) was designed to have the same base composition as the ASCH oligonucleotide (Fig. 4-3C).

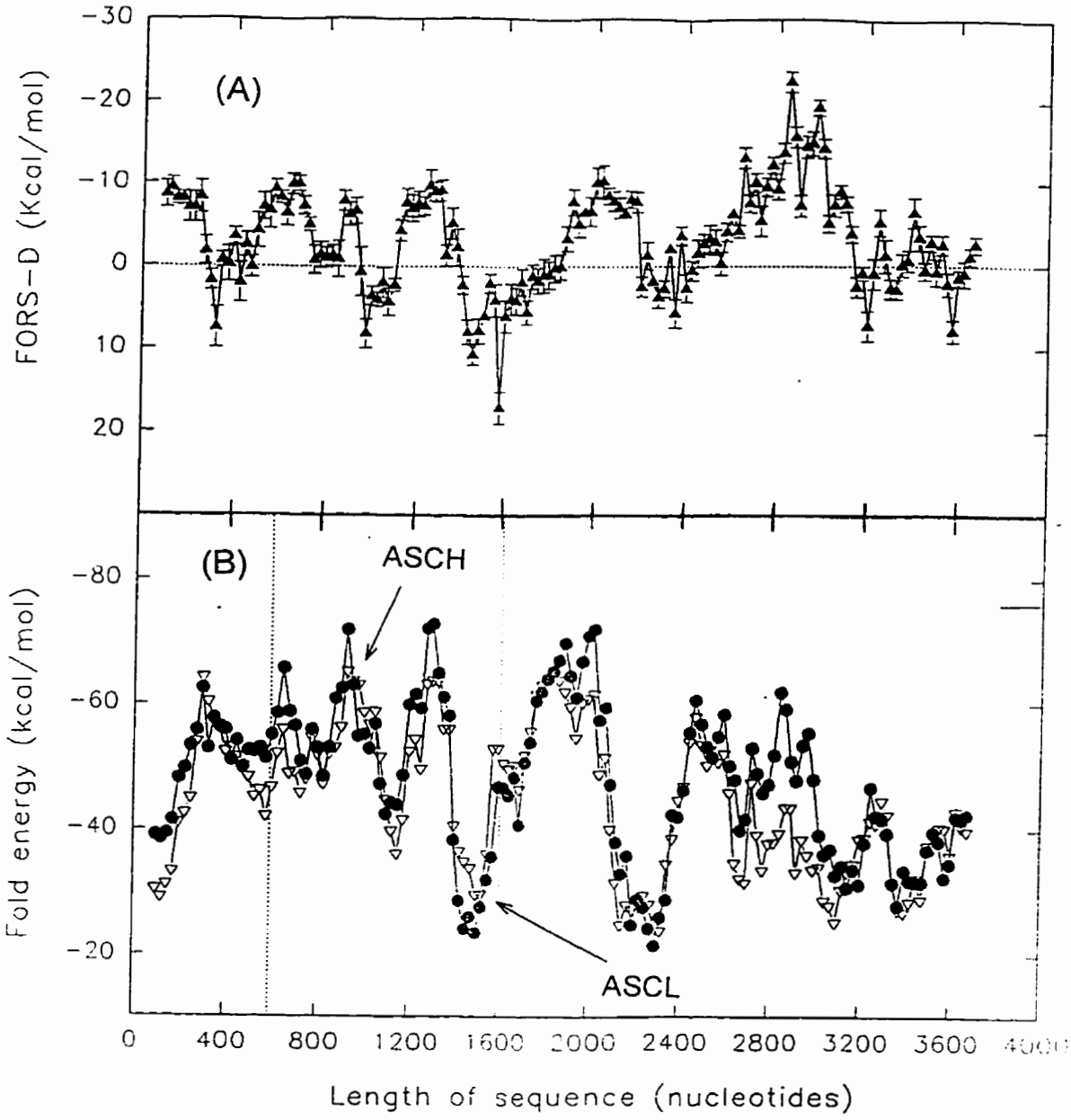
G0S3/FOSB mRNA and protein levels transiently increase in response to Con-A

Confirming the affects of Con-A on *G0S3/FOSB* mRNA expression, RT-PCR analysis showed that *G0S3/FOSB* mRNA levels increase in response to Con-A, peaking at 1 hr and then declining (Fig. 4-4A). Immunoblot analysis demonstrated that G0S3/FOSB protein levels also increase and are maximal at 2 hr after Con-A addition, and then decline (Fig. 4-4B).

G0S3/FOSB protein levels are not significantly less in cultures treated with ASCH or ASCL oligonucleotides

Having established a G0S3/FOSB protein induction profile, our next aim was to

Figure 4-3. **Fold energy minimization values (FONS, FORS-M) and differences (FORS-D) for *G0S3/FOSB* mRNA.** Details are as outlined in Fig. 4-1. The mean minimum energy value for a set of 10 randomized sequences (FORS-M) is plotted with the corresponding FONS value (B). The differences between FORS-M values and the corresponding FONS values are plotted (FORS-D ± standard error) (A). Regions of *G0S3/FOSB* mRNA to be targeted by antisense oligonucleotides were selected on the basis of high and low base composition-determined stem-loop potential. Sequences of the antisense oligonucleotides targeted to regions of high (ASCH) and low (ASCL) base composition-determined stem-loop potential as well as a shuffled (SH) oligonucleotide are shown (C). Arrows indicate the precise regions to which the oligonucleotides are targeted (B).



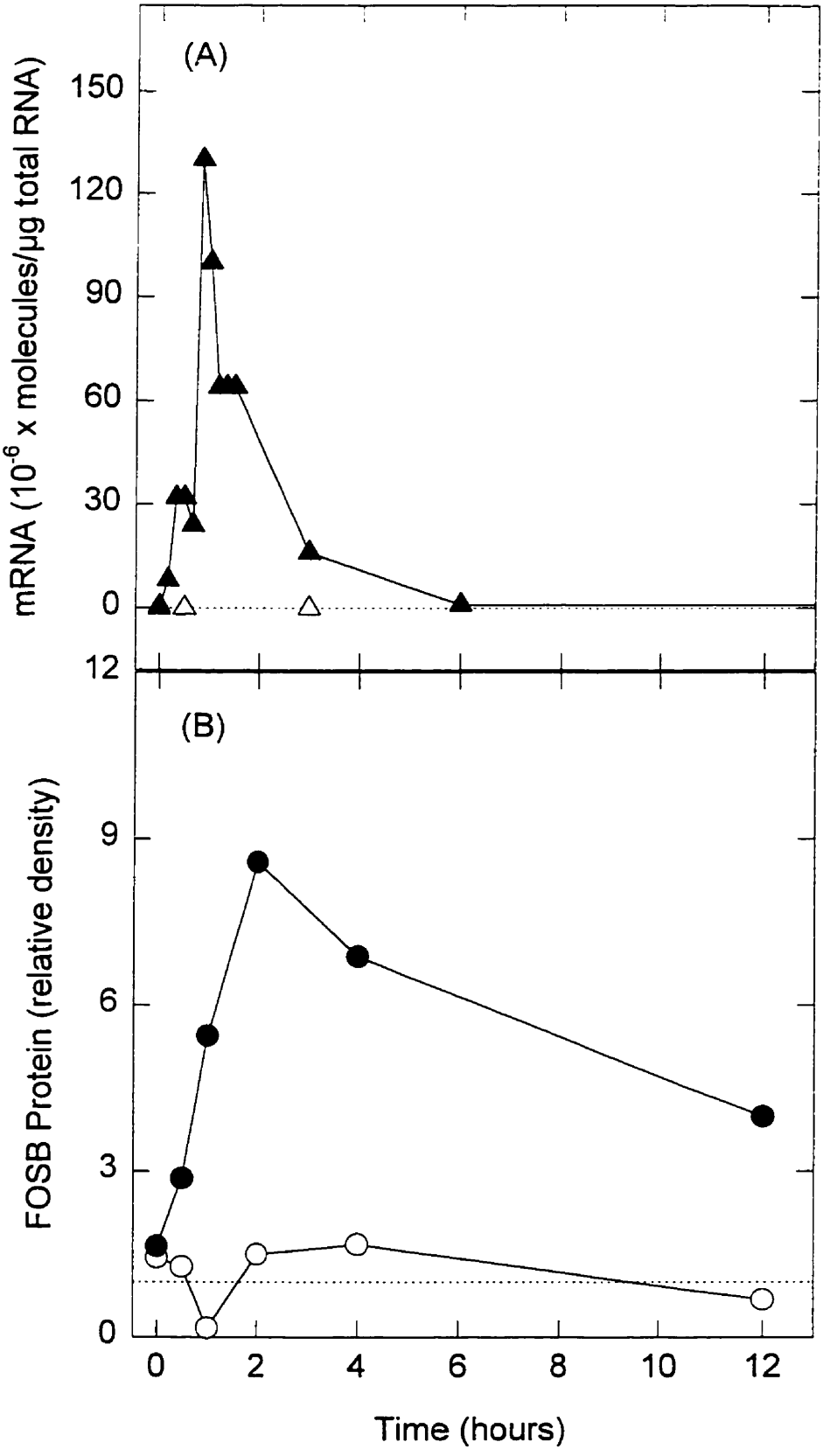
(C)

ASCH 5'- GCT GTA GCC ACT CAT -3'

ASCL 5'- GAC AAA CGA AGA AGT -3'

SH 5'- CCG AGA CCT GAT CTT -3'

Figure 4-4. **Time course of effects of Con-A on mRNA and protein levels of *G0S03/FOSB* in human blood mononuclear cells.** Figure 4-4A and 4-4B illustrate the results of one representative experiment, however these experiments were repeated at least three times with different human donors.Human blood mononuclear cells were preincubated for 24 hr before addition of Con-A. Filled symbols indicate cultures with Con-A. Each point represents a single value. The effects of Con-A on *G0S3/FOSB* mRNA levels (A), and on relative levels of G0S3/FOSB protein are shown (B). The horizontal dashed line in (B) corresponds to the G0S3/FOSB protein level in freshly isolated cells, designated as 1.0 scanning densitometry units.


(A)
150
120
90
60
30
0
mRNA (10^{-6} x molecules/µg total RNA)
12
(B)
9
6
3
0
FOSB Protein (relative density)
0
2
4
6
8
10
12
Time (hours)

examine the effects of varying concentrations of the antisense and shuffled oligonucleotides on G0S3/FOSB protein levels. Figure 4-5 illustrates the results of one representative experiment in which human blood mononuclear cells were pretreated with varying concentrations of ASCH, ASCL or SH oligonucleotides for 1.5 hr prior to Con-A addition. Cells were harvested at 2 hr following Con-A treatment. In 4 independent experiments (4 different human donors), the inhibition of G0S3/FOSB protein was not found to be significant in cultures treated with either ASCH oligonucleotide or ASCL oligonucleotide relative to cultures treated with Con-A alone, at any of the concentrations tested.

However, a significantly lower G0S3/FOSB protein level was found in cultures pretreated with ASCH oligonucleotide relative to those pretreated with ASCL oligonucleotide at one particular oligonucleotide concentration (0.5 nM; mean of 4 experiments = 36.5%, $P = 0.02$). To this extent, our presumption that ASCL would be more effective was contradicted.

Antisense oligonucleotides do not significantly affect G0S03/FOSB mRNA levels

We then assessed the effects of oligonucleotides on *G0S3/FOSB* mRNA levels in Con-A-stimulated cultures. Figure 4-6A illustrates a representative RT-PCR profile, and figure 4-6B graphically summarizes the results of 3 independent experiments. There was found to be no significant difference in *G0S3/FOSB* mRNA levels in Con-A-induced cultures pretreated with either ASCH, ASCL or SH oligonucleotide, relative to those treated with Con-A alone. At all oligonucleotide concentrations examined there was also found to be no statistically significant difference in *G0S3/FOSB* mRNA levels in ASCH oligonucleotide-

Figure 4-5. **Effects of *G0S03/FOSB* antisense and control oligonucleotides on *G0S03/FOSB* protein levels in human blood mononuclear cells.** This figure illustrates a bar graph representation of one experiment. This experiment was repeated with 4 different human donors. Cells were preincubated for 24 hr prior to the addition of either *G0S3/FOSB* ASCH (first bar; filled), ASCL (second bar; filled) or SH (third bar; not filled) oligonucleotides. After an additional 1.5 hr incubation, Con-A (200 μg/ml) was added. Cells were harvested 2 hr after Con-A addition for immunoblot detection of G0S3/FOSB protein.

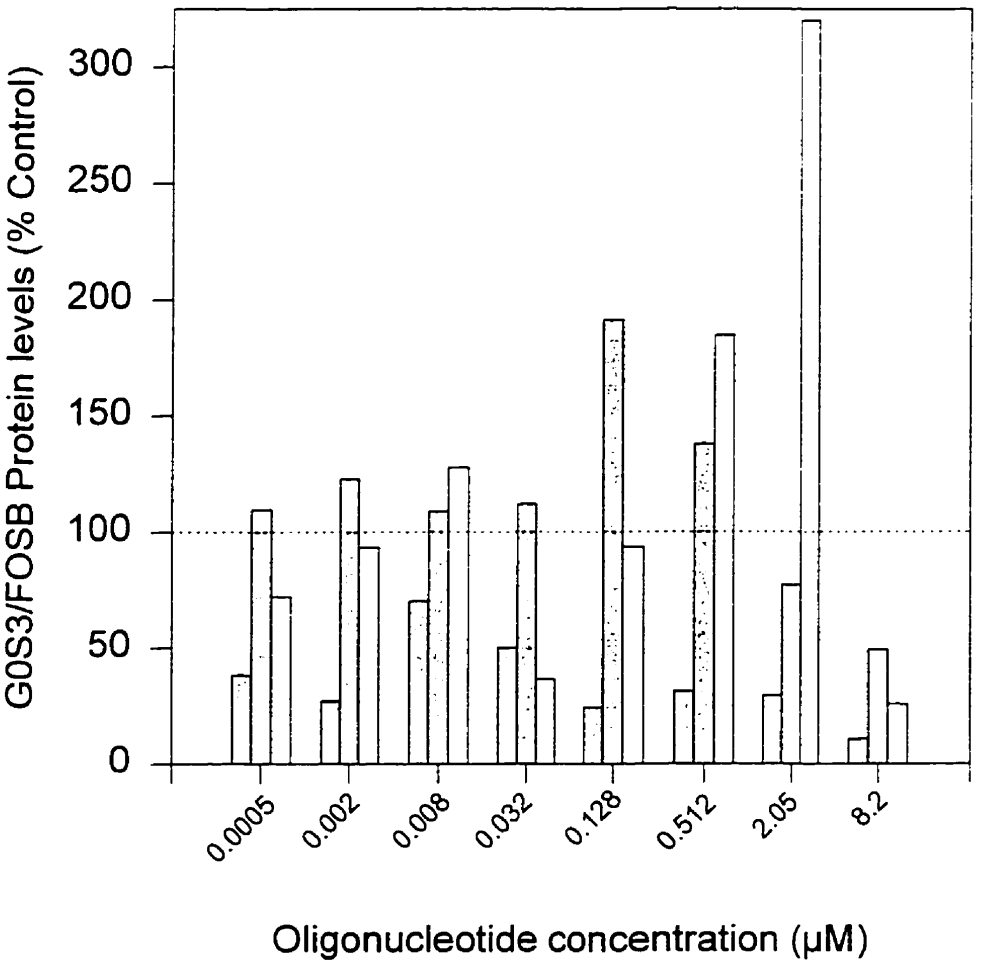
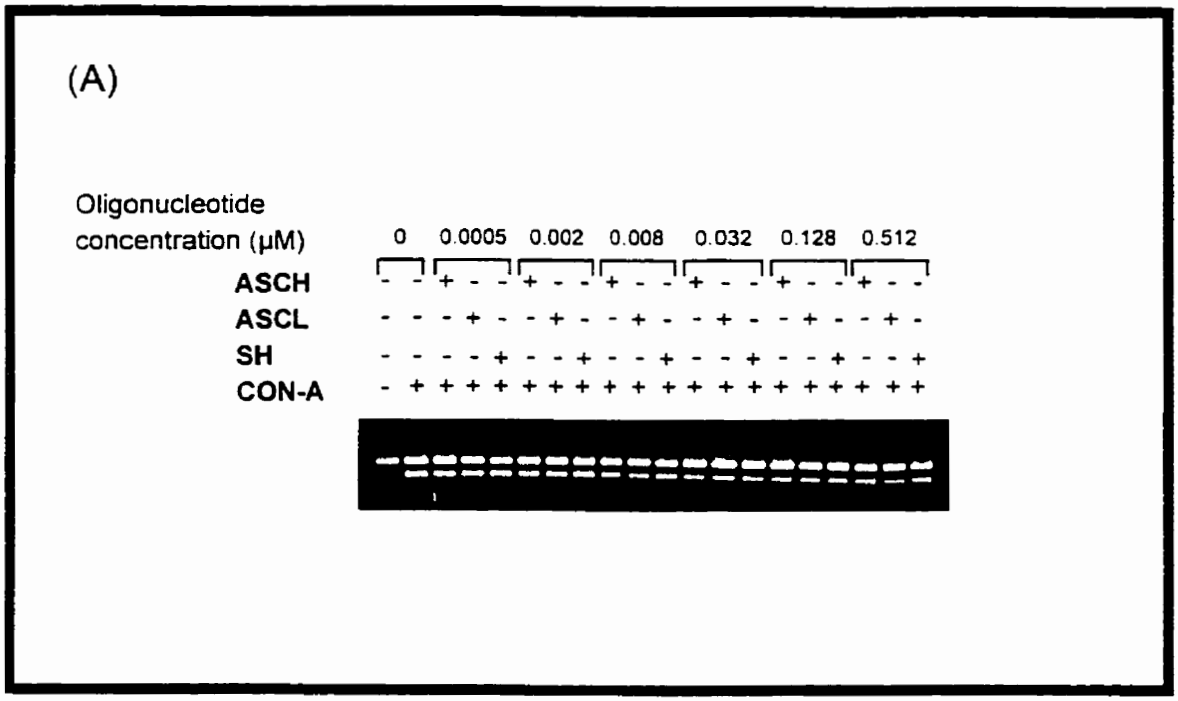
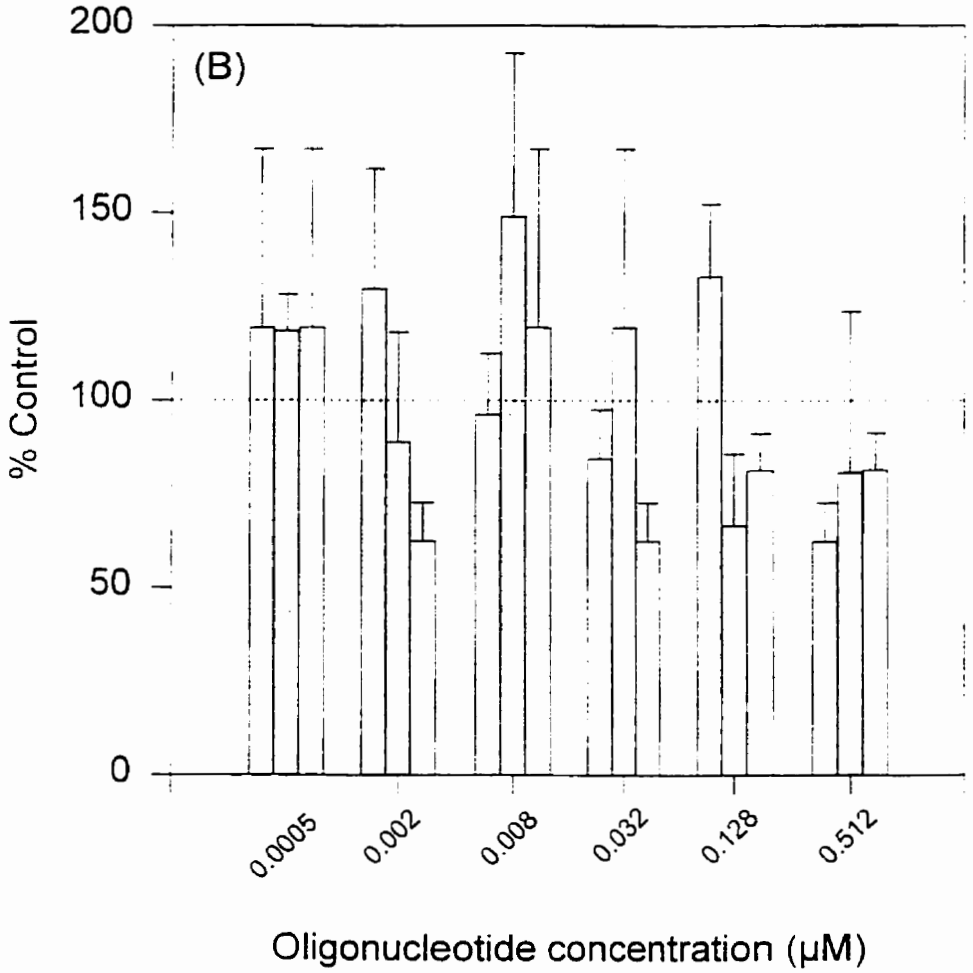

G0S3/FOSB Protein levels (% Control)
300
250
200
150
100
50
0
0.0005
0.002
0.008
0.032
0.128
0.512
2.05
8.2
Oligonucleotide concentration (μM)

Figure 4-6. **Absence of an effect by *G0S03/FOSB* antisense and control oligonucleotides on *G0S03/FOSB* mRNA levels in cultures of Con-A-stimulated blood mononuclear cells.** Cells preincubated for 24 hr prior to the addition of either ASCH (first bar; filled), ASCL (second bar; filled) or SH (third bar, not filled) oligonucleotide. After an additional 1.5 hr incubation, Con-A (200 μg/ml) was added. Cells were harvest 1 hr after Con-A addition. The upper photograph (A) shows an mRNA profile using RT-PCR and (B) illustrates a summary of the quantitation of *G0S3/FOSB* mRNA levels. Each bar graph illustrates the mean level of *G0S3/FOSB* mRNA from three independent experiments and is expressed as a percentage of control levels (duplicate samples with no oligonucleotides added) (± SEM).


(A)
Oligonucleotide concentration (μM)
0 0.0005 0.002 0.008 0.032 0.128 0.512
ASCH
ASCL
SH
CON-A

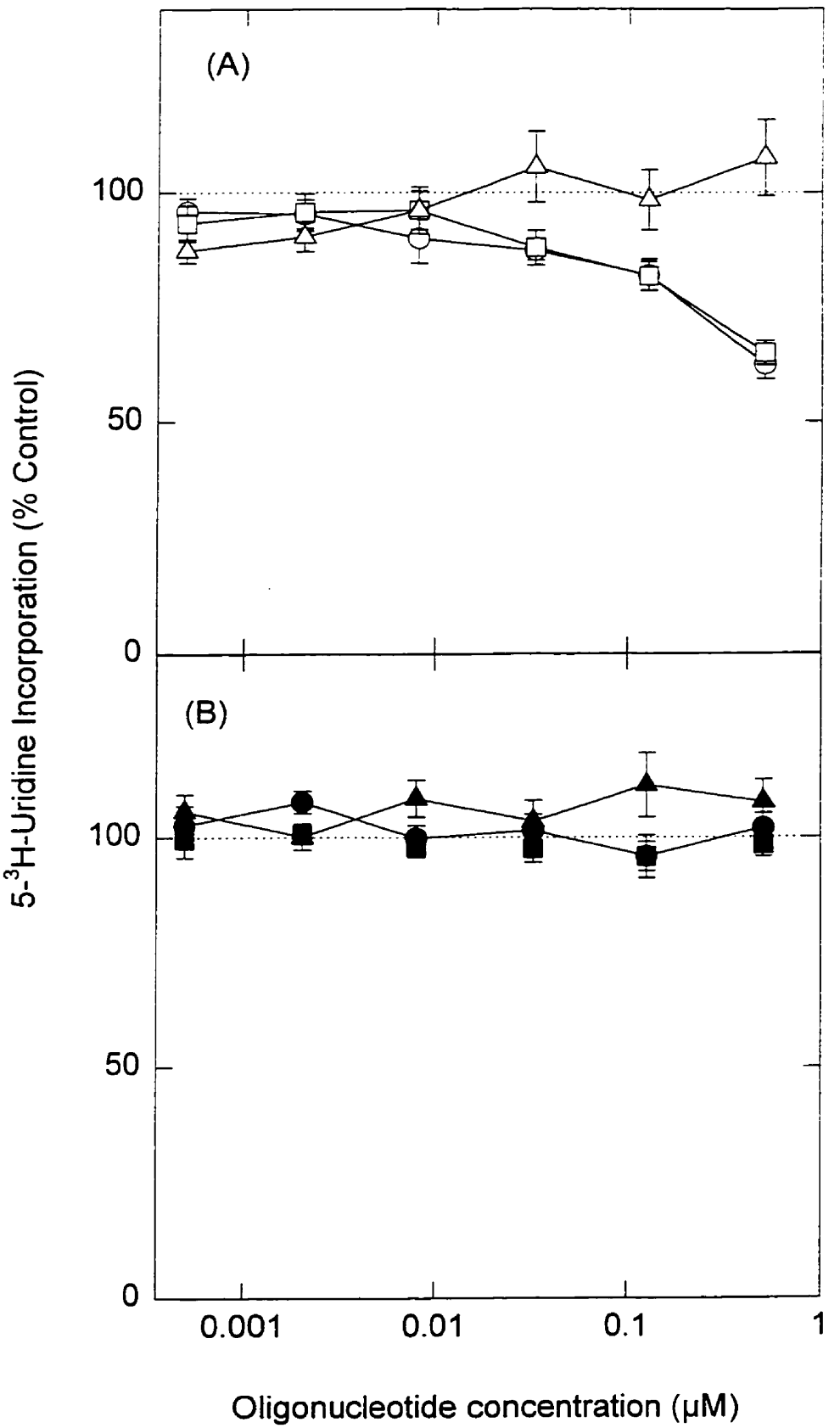

(B)
200
150
100
50
0
% Control
0.0005
0.002
0.008
0.032
0.128
0.512
Oligonucleotide concentration (μM)

treated cultures relative to those treated with ASCL or SH oligonucleotide.

Specific inhibition of RNA synthesis by ASCH and ASCL oligonucleotides is noted in control cultures but not in Con-A-stimulated cultures

We next tested the effects of oligonucleotides on RNA synthesis by measuring the incorporation of [^{3}H]uridine in human blood mononuclear cells. The results of 3 independent experiments in which cultures were not stimulated with Con-A are summarized in Figure 4-7A. Here we note that both ASCH oligonucleotide and ASCL oligonucleotide concentrations between 32 nM and 0.512 μM inhibit the incorporation of [^{3}H]uridine. Cultures treated with 32 nM, 128 nM and 0.512 μM of ASCH oligonucleotide demonstrated inhibition of [^{3}H]uridine incorporation that was 12.7% ± 2.1% (P<0.0001), 18.1% ± 3.5% (P<0.0001) and 37.5% ± 3.2% (P<0.0001), respectively, less than that of cultures not treated with oligonucleotides. Cultures similarly treated with ASCL oligonucleotide demonstrated inhibition of [^{3}H]uridine incorporation that was 12.1% ± 3.8% (P<0.01), 18.2% ± 3.1% (P<0.0001) and 35.0% ● 2.6% (P<0.0001), respectively, less than that of cultures not treated with oligonucleotide. There was no significant difference in the extent of inhibition of [^{3}H]uridine incorporation between ASCH oligonucleotide- and ASCL oligonucleotide-treated cultures, again contradicting our presumption that ASCL would be more effective. The inhibition noted is specific in that the shuffled oligonucleotide, which is not complementary to *G0S3/FOSB*, did not show the inhibition. Moreover, inhibition was only noted in control cells without Con-A, not in Con-A-treated cultures (Fig. 4-7B).

Figure 4-7. **Effects of *G0S03/FOSB* antisense and control oligonucleotides on RNA synthesis.** Cells (1.5 x 10^6/ml) were preincubated for 24 hr prior to the addition of Con-A. Cells were pretreated with varying concentrations of ASCH, ASCL or SH oligonucleotide for 1.5 hr. Cells were then either not treated (A) or treated (B) with Con-A and then incubated for an additional 22 hr before harvesting. Circles, ASCH oligonucleotide; squares, ASCL oligonucleotide; triangles, control SH oligonucleotide. Levels of radioactivity are expressed as a percentage of the levels found in samples not treated with oligonucleotides in the absence of Con-A (A) and in the presence of Con-A (B). Each point is the mean value of three independent experiments (± SEM). In each of the three experiments, triplicate cultures were assayed for each oligonucleotide concentration examined. The horizontal dashed lines in (A) and (B) reflect 100% control levels (sample not treated with oligonucleotides).


(A)
100
50
0
(B)
100
50
0
0.001
0.01
0.1
1
5-3H-Uridine Incorporation (% Control)
Oligonucleotide concentration (μM)

DISCUSSION

In our computational analysis of nine studies carried out in cultured cells (83, 182-188), there were no significant trend noted between the extent of inhibition and FONS, FORS-M or FORS-D values (Table 4-1). However, in analyzing the five studies carried out in cell-free extracts (82, 189), *all* five studies consistently showed a negative linear relationship between the extent of inhibition and FORS-M value (mean slope = -1.82 ± 0.58, P = 0.014; Table 4-2). This finding suggested that regions of mRNA with *low* base composition-determined stem-loop potential would be the most effective target sites for antisense oligonucleotide-mediated inhibition. Although our inhibition studies of *G0S* genes were going to be carried out in cultured cells, and not cell-free extracts, we still decided to use this one consistent trend from our bioinformatic analysis to assist us in designing antisense oligonucleotides targeted to *G0S3/FOSB* mRNA. We therefore selected antisense oligonucleotides targeted to regions of *G0S3/FOSB* mRNA with *high* (ASCH) and *low* (ASCL) base composition-determined stem-loop potential, presuming that the latter would be more effective in inhibiting the expression of *G0S3/FOSB*.

When cells were treated prior to Con-A stimulation with various concentrations of these antisense oligonucleotides, the resulting effects varied. Although there is some evidence of G0S3/FOSB protein inhibition in Con-A-stimulated cultures pretreated with 0.5 nM ASCH oligonucleotide, relative to cultures with Con-A alone, this inhibition was not found to be significant (Fig. 4-5). Contradicting the one consistent trend noted in our bioinformatic analysis, cultures pretreated with ASCH oligonucleotide were found to show significantly less G0S3/FOSB protein levels compared to cultures pretreated with ASCL

oligonucleotide at the very low oligonucleotide concentration of 0.5 nM (mean of 4 experiments = 36.5%, P=0.02). This result suggests that mRNA regions with *high*, not low, base composition-determined stem-loop potential should be targeted by antisense oligonucleotides for effective inhibition.

We found that pretreatment of control cultures (no Con-A added) with either ASCH oligonucleotide or ASCL oligonucleotide results in an inhibition of RNA synthesis, as measured by [^{3}H]uridine incorporation (Fig. 4-7). This finding suggests that we may be inhibiting the low basal levels of G0S3 protein expression that is normally found in control cultures (28). By inhibiting G0S3/FOSB, we may be removing a component required for subsequent RNA synthesis, and thus, note a decrease in [^{3}H]uridine incorporation. Unfortunately, it was not possible to verify inhibition of *G0S3/FOSB* expression by assaying the levels of *G0S3/FOSB* mRNA or GOS3/FOSB protein in control cultures pretreated with oligonucleotides. The reason for this is that the very low basal levels of *G0S3/FOSB* mRNA and G0S3/FOSB protein were barely visible as bands on agarose gels and western blot membranes, respectively, from these unstimulated cultures. Therefore, inhibition by ASCH or ASCL would have been extremely difficult to assess. In future, one could assay the protein levels of stromelysin and collagenase, which are suggested targets of FOSB (197), and assess if there is any down regulation in these control samples.

The maximum inhibition of [^{3}H]uridine incorporation by the ASCH and ASCL oligonucleotides were 37.5 % ± 3.2 % ($P < 0.001$) and 35.0 % ± 2.6 % ($P < 0.001$), respectively. This raises the possibility that either the concentration required to achieve 100% inhibition was not found, or the requirement for G0S3/FOSB is not absolute (ie. perhaps

G0S7/FOS can compensate) (198). Future studies testing higher concentrations of oligonucleotide concentrations and assessing the up-regulation of FOS family members, such as G0S7/FOS, will address this question.

The inhibition of RNA synthesis noted in control samples was not seen in cultures treated with Con-A (Fig. 4-7). This suggests that the transient induction of *G0S3/FOSB* by Con-A results in high mRNA and protein levels that cannot be reduced to limiting concentrations by the antisense oligonucleotides. Future studies, testing a higher concentrations of oligonucleotide may identify a concentration that can more effectively inhibit the expression of *G0S3/FOSB*. Our results suggest that there is a low basal level of expression in a large proportion of our cultured cells, rather than a high level of expression in a minority population. In the latter case we envisage that it would be difficult to achieve an intracellular oligonucleotide concentration sufficient to inhibit G0S3/FOSB-dependent uridine incorporation into RNA.

In retrospect, a target such as *G0S3/FOSB*, present at very high transiently-induced levels, may have been a poor choice for our antisense oligonucleotide inhibition studies. The availability of a commercial anti-FOSB antibody enabling us to assay G0S3/FOSB protein levels following treatment of cells with oligonucleotides made it seem a good choice initially. Future production of antibodies for other members of the *G0S* family of genes will enable similar studies on the expression of these genes.

Our bioinformatic analysis proved inconclusive. The one consistent trend that was noted in this study was contradicted both in the inhibition of G0S3/FOSB protein levels and in the inhibition of [^{3}H]uridine incorporation. Our inability to find a consistent predictor of

effective antisense oligonucleotides suggests that our knowledge of factors influencing RNA structure may be incomplete. Computational analysis of not only target mRNAs, but also genomic sequences that gives rise to these mRNAs may further our understanding of RNA structure and could potentially unveil factors that may be of use in designing effective antisense oligonucleotides. This is the subject of the next chapter.

CHAPTER 5

Viruses Profoundly Committed to Latency (HTLV-1, Epstein Barr) Disobey the Chargaff Difference Transcription Direction Rule.

ABSTRACT

In the 1960's Szybalski showed that mRNA-synonymous strands of DNA have purine-rich clusters, and Chargaff presented his second parity rule that, to a close approximation, %A = %T and %C = %G for single DNA strands. Extending these observations, it was found that small deviations from the rule (%purines > % pyrimidines in mRNA-synonymous strands) allow determination of transcription direction for the majority of genes in many species (199). The purine clusters in an mRNA correspond to the loop domains of potential stem-loop structures, which, by virtue of being enriched with non-complementary bases, should avoid loop-loop "kissing" interactions with other mRNAs in the same cell. However, most genes of CG-rich viruses with a high commitment to latency (HTLV-1, Epstein-Barr) disobey the transcription direction rule (they have %pyrimidines > %purines in mRNA-synonymous strands). Thus, the viruses would have pyrimidine clusters in the loop domains of potential RNA stem-loop structures. By following this alternative rule, they are mimicking a car that is "driving on the wrong side of the road", and thus, they invite sense-antisense RNA "collisions", which could trigger the interferon response and increase MHC protein expression. However, the only gene transcribed during the "EBNA-1 only program" of viral latency obeys the transcription direction rule (%purines > %pyrimidines in the mRNA synonymous strand). This is achieved by purine-loading the

mRNA, which forces the protein to accept a long simple-sequence domain that may not affect its functional domains.

INTRODUCTION

The "crowded cytosol" (200) is likely to be physically optimized for tRNA-mRNA interactions, which must occur rapidly and accurately for efficient protein synthesis. This involves interactions between anticodon loops of tRNAs and codons on mRNAs. Tomizawa (201) has shown how folded single-stranded RNAs can engage in reversible loop-loop interactions (referred to as "kissing"), which in the case of complementary sense and antisense RNAs can lead to formation of a hybrid duplex (202).

It has been proposed that a cytosolic environment which promotes tRNA-mRNA interactions would also be highly conducive to mRNA-mRNA interactions, so that there might have been an evolutionary selection pressure militating against such distracting loop-loop interactions (199). This pressure would have selected for mutations in mRNAs such that the loops of mRNAs which must coexist in a common cytosol became enriched either in the same base, or in particular non-Watson-Crick-pairing bases (because a loop only "kisses" a loop containing complementary bases). All the mRNAs in a given cytosol would therefore tend to obey the *same* Chargaff difference transcription direction rule (i.e. "drive on the same side of the road"). Do the mRNAs of an intracellular pathogen obey the same rule?

We have found that some viruses do not obey the transcription direction rule (199). We here explore the hypothesis that the extent to which viruses obey this rule may be related to the degree to which a virus is committed to a latency strategy. Our results may explain

why double stranded RNA is such a powerful inducer of interferon (203), and provides a possible mechanism for intracellular self/not-self discrimination to allow selective MHC presentation of foreign peptides (200, 204, 205).

MATERIALS AND METHODS

Chargaff Difference Analysis

Each sequence was examined using 1 kb windows which were moved along the sequence in steps of 0.1 kb. Base composition (C, G, A, and T) was determined in these 1 kb windows using a unix script file that was implemented on the Silicon Graphics Computer maintained by Base4 BioInformatics Inc., Mississauga. A, C, G, and T refer to the quantity of the corresponding base in each window. Chargaff differences for the W bases were calculated either as (A-T)/W, or as (T-A)/W, or (for absolute values) as $_{\Delta}W/W$. These values were expressed as percentages. W is the sum of the W bases (W=A+T), and $_{\Delta}W$ is the absolute value of the difference between the number of W bases ($_{\Delta}W = |A-T|$). Chargaff differences for the S bases were calculated either as (C-G)/S, or (for absolute values) as $_{\Delta}S/S$, and expressed as percentages. S is the sum of the S bases (S=C+G), and $_{\Delta}S$ is the absolute value of the difference between the number of S bases ($_{\Delta}S = |C-G|$).

Statistics

The Wilcoxon signed ranks test, a nonparametric alternative to the t test, was carried out as outlined (110). The Wilcoxon procedure detects shifts in location of probability distributions. Probability that the values were not significantly different from zero (t test) was

examined as previously described (Chaper 2 Materials and Methods)

RESULTS AND DISCUSSION

Three Retroviruses Follow the Normal Transcription Rule

In many genomes transcription directions vary so that, while total base compositions of single-stranded DNA reflect Chargaff's second parity rule, Chargaff differences for leftward transcribing ORFs (e.g. C>G) tend to cancel out the differences for rightward transcribing ORFs (e.g. G>C). However, retroviral genomes are transcribed entirely to the right, and mere examination of total base composition can illustrate some major points.

Table 5-1 compares the "top" strands of four retroviruses whose (C+G) percentages vary from 39.2% (Simian foamy virus-1; SFV-1; 206), to 54.4% (Rous Sarcoma virus; RSV; 207). Deviations from Chargaff's second rule (calculated from total base compositions) tend to be extreme in retroviruses, and three of the genomes obey the transcription direction rule for rightward transcription (purines>pyrimidines). For AT-rich genomes (SFV-1, HIV-1; 208), A>T and G>C with the W (weak) bases providing the largest deviation from Chargaff's second rule. Similarly, for the CG-rich RSV genome, A>T and G>C, with the S (strong) bases providing the largest deviation. Calculations for local regions in the sequences (1 kb windows) show that these purine-rich patterns are sustained throughout most of the sequences, particularly in the case of the base pairs with the largest deviations from Chargaff's second rule.

Table 5-1. **Chargaff differences of retroviral genomes (which transcribe to the right)**

Virus*	Base composition					Chargaff differences (%)†	
	W bases		S bases				
	A	T	C	G	(C+G)%	(A-T)/W	(C-G)/S
SFV-1	4195	3696	2480	2601	39.2	A>T (6.32)	G>C (-2.38)
HIV-1	3411	2163	1772	2373	42.6	A>T (22.39)	G>C(-14.50)
HTLV-1	1983	1951	2932	1534	53.2	A>T (0.81)	C>G (31.30)
RSV	2216	2035	2362	2704	54.4	A>T (4.26)	G>C (-6.75)

* SFV-1, simian foamy virus type 1 (Genbank accession number X54482); HIV-1, human immunodeficiency virus 1 (K03455); HTLV-1, human T cell leukaemia virus 1 (D13784); RSV, Rous sarcoma virus (D10652).

† Values were calculated for the entire genome, which was not split into sub-windows.

A, T, C, and G refer to numbers of bases. W = A + T; S = C + G.

CG-rich HTLV-1 Does Not Obey Transcription Direction Rule

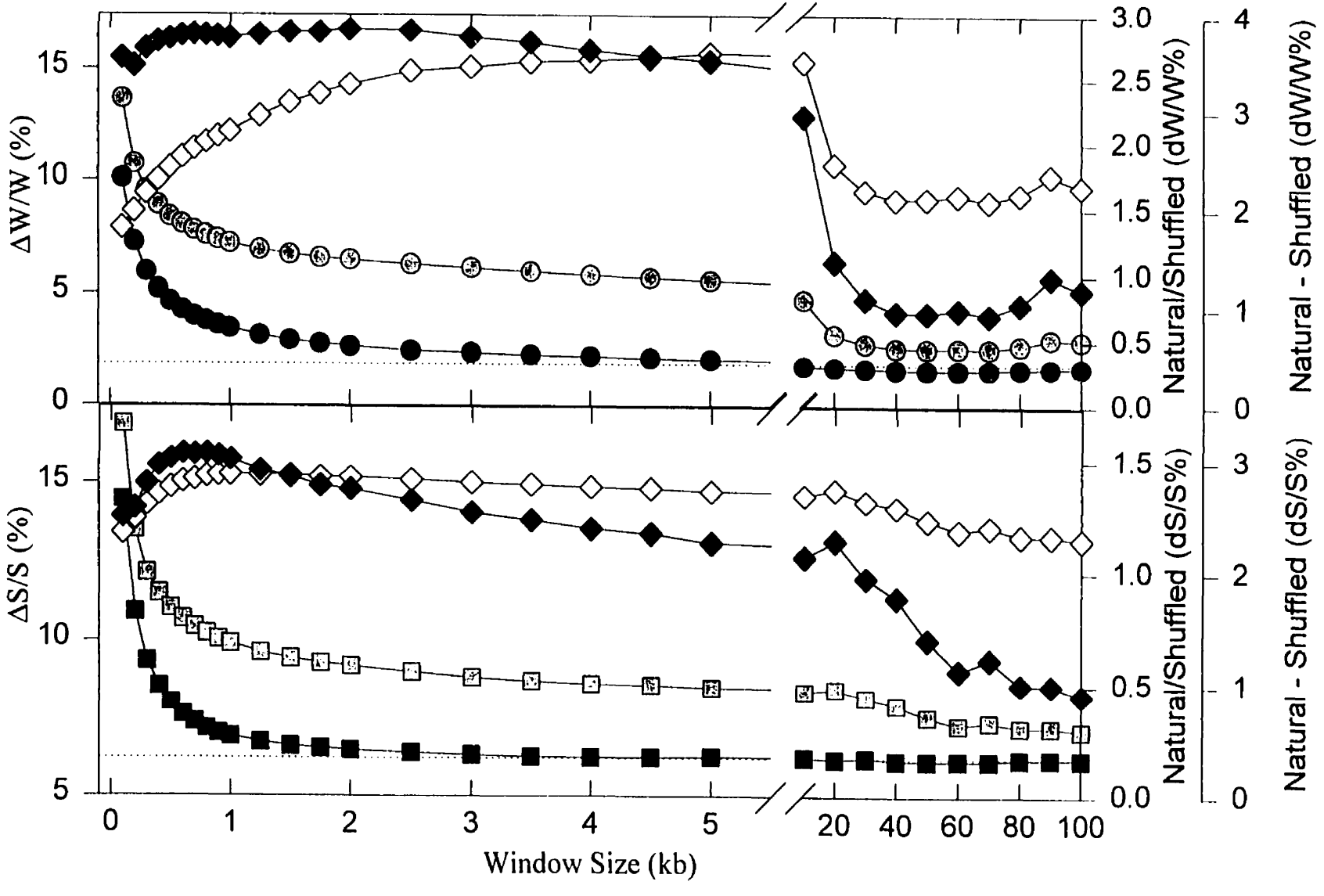
A cell with an mRNA population of one particular loop type might place constraints on artificial expression vectors and intracellular pathogens ("non-self"), which may need to avoid encoding mRNAs capable of pairing with host mRNAs. However, by obeying the Chargaff difference transcription direction rule, the above three retroviral pathogens appear to have transcripts that "drive on the same side of the road" as most mRNAs of their hosts and thus, would not cause conflict. This also applies to SV40 and polyoma viruses (209), and to vaccinia virus (199). In contrast, human T cell leukaemia virus (HTLV-1, 210) seems to invite disaster. It is CG-rich, yet C>G. There is only a weak tendency for compliance with the Chargaff difference direction rule with respect to the W bases (Table 5-1). Transcripts of HTLV-1 therefore tend to have loops enriched with pyrimidines, opposite to the state of host cell transcripts.

We suggest that this relates to a greater commitment of HTLV-1, than of the other viruses, to the latent state; in this state, most mRNAs would not be expressed in the cytosol, and so would not be available to interact with host mRNAs. In contrast to individuals infected with HIV-1, most individuals infected with HTLV-1 remain asymptomatic and live normal lives. Furthermore, HTLV-1 is likely to transfer between individuals when integrated in host DNA within intact cells, and virions alone show low infectivity. An explanation for this may be that when in the cytosol viral RNA is confronted with host RNAs that are all "driving on the normal side of the road". Why then does HTLV-1 not obey the transcription direction rule?

CG-rich Herpes Viruses Also Do Not Obey Transcription Direction Rule

To examine this further, we carried out Chargaff difference studies on other viruses which are committed to the latent state. *Herpes simplex*-related viruses permanently infect many individuals in their host species, who are often asymptomatic (211-214). The Chargaff difference values give an indication of the extent of deviation from Chargaff's second rule for the A + T bases (W), and the G + C bases (S). Chargaff differences were determined for increasing window sizes both in the natural sequence, and in a shuffled sequence of *Herpes virus saimiri* (HVS; Fig. 5-1). The shuffled sequence consists of the same base composition as the natural sequence, but the sequence was generated by randomizing the natural sequence using the GCG program SHUFFLE (215). Figure 5-1 illustrates absolute Chargaff difference values, for W and S bases, plotted versus the size of the sequence windows. With increasing window size, average Chargaff differences for both the natural and shuffled sequences decrease, approaching the Chargaff difference value of the entire segment (horizontal dotted lines). Values for the natural sequence were found to exceed those of the shuffled sequence. This suggests that there is some evolutionary pressures on the base order which favour the formation and maintenance of Chargaff differences. Values for the natural and shuffled sequence were compared either as a ratio (open diamonds), or by difference (filled diamonds). The size of the window at which Chargaff differences for the natural and shuffled sequences maximally diverge depends on the method used. In the case of W bases, divergence by difference was found to be maximal with 1 kb windows. Divergence by ratio was found to be very high with 1 kb windows, which increased slightly at window sizes greater than 1 kb. This continued until maximal divergence was reached at approximately

Figure 5-1. **Variation of average absolute Chargaff difference values with varying window size in the nucleotide sequence of *Herpes virus saimiri* (HVS).** Windows of varying size were moved along the sequence in steps of 100 nucleotides, and base compositions were determined in each window. Absolute Chargaff differences were calculated for (a) as ΔW/W and for (b) as ΔS/S and expressed as a percentages. Average Chargaff differences for each window size are plotted either as grey circles, squares (natural sequence), or as black circles, squares (shuffled sequence). Open diamonds refer to the ratio of these values (the average Chargaff difference for the natural sequence divided by the average Chargaff difference for the shuffled sequence). Filled diamonds refer to the difference between these values. The horizontal dotted lines indicate Chargaff differences for the entire sequence (i.e. the largest possible window, of which there is only one copy).


ΔW/W (%)
ΔS/S (%)
Natural/Shuffled (dW/W%)
Natural - Shuffled (dW/W%)
Natural/Shuffled (dS/S%)
Natural - Shuffled (dS/S%)
Window Size (kb)
0
1
2
3
4
5
20
40
60
80
100
0.0
0.5
1.0
1.5
2.0
2.5
3.0
5
10
15

3.5 kb. For the S bases, maximal divergence by difference and ratio was found with 1 kb and 0.8 kb windows, respectively. Analysis of other *Herpes virus* family members gave similar findings. Our results suggest that evolutionary pressures on base order, which favour the formation and maintenance of Chargaff differences, are maximal with a 1 kb window.

Table 5-2 shows that, like HTLV-1, CG-rich members of the herpes virus family (EBV, HSV) also do not follow the normal transcription direction rule. This applies strongly to the S bases (e.g. C>G when transcription is to the right), and with marginal significance to the W bases (e.g. T>A when transcription is to the right).

For most genes of AT-rich members of the herpes virus family (HVS, VZV), the W bases follow the normal transcription direction rule when transcription is to the right (A>T), whereas the S bases follow the "HTLV-1 rule" (C>G). When transcription is to the left, both the W and S bases follow the normal Chargaff difference transcription direction rule (pyrimidines>purines), but this is most evident in the case of the S bases. On balance, like HIV-1 (Table 5-1), the AT-rich herpes viruses seem to follow the normal rule.

Normal Transcription Direction Rule Obeyed in "EBNA-1 Only Program"

Intriguingly, an "exception" appears to "prove the rule". Figure 5-2 shows Chargaff difference analysis of the section of the CG-rich Epstein-Barr virus (EBV) genome from which a major latency-associated transcript (encoding EBNA-1 protein; 216) is derived. Whereas the neighbouring genes follow the "HTLV-1" rule (C>G when transcription is to the right; G>C when transcription is to the left), the rightward transcribing gene encoding EBNA-1 protein follows the normal transcription direction rule (G>C; A>T), and very

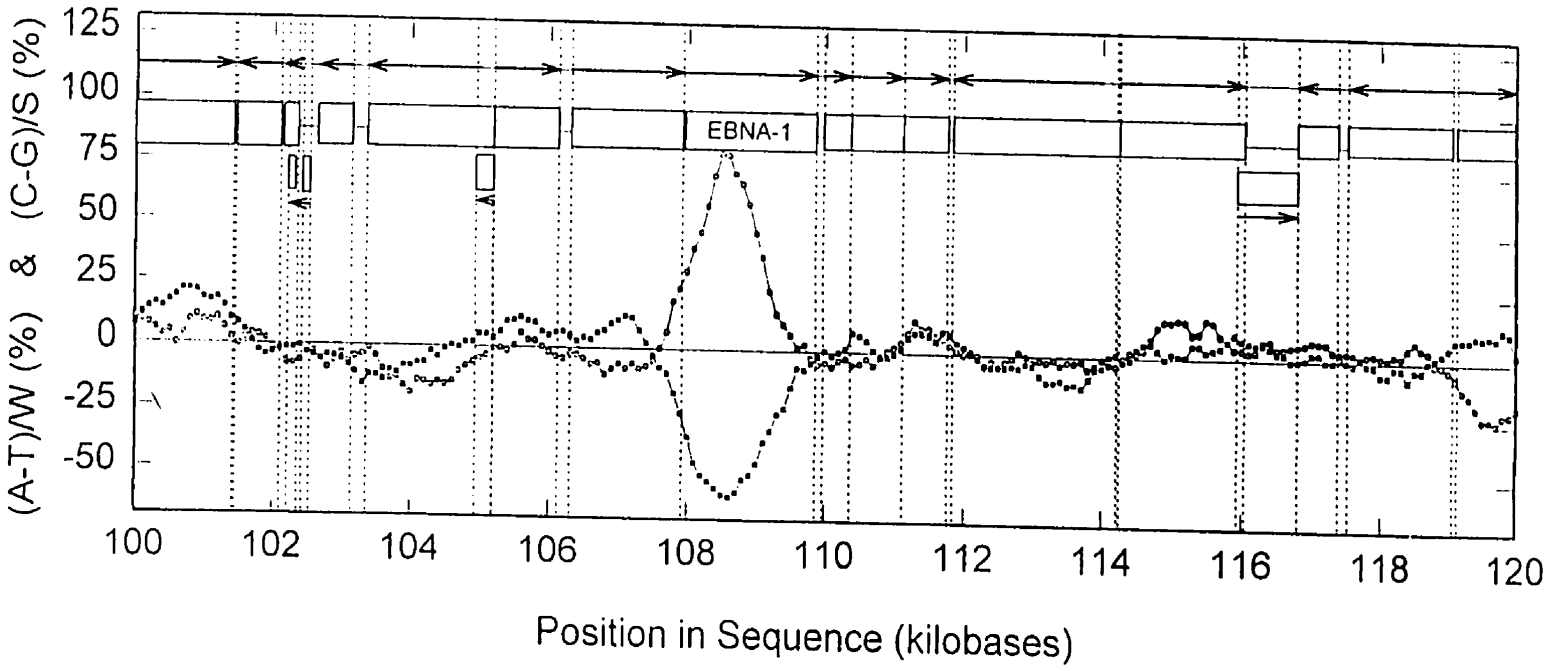
Table 5-2. **Average Chargaff differences for leftward and rightward transcribing ORFs of various herpes-related viruses**

Difference formula*		(A-T)/W %		(C-G)/S %	
Direction of transcription		To left	To right	To left	To right
Virus†	C+G (%)				
HVS	35.0	T>A 22:15 (P=0.020) -3.31±1.56 (P<0.025)	A>T 35:5 (P<0.00003) 4.61±0.77 (P<0.0005)	C>G 28:9 (P=0.00016) 6.95±1.79 (P<0.0005)	C>G 28:12 (P=0.0035) 4.02±1.43 (P<0.005)
VZV	46.1	T>A 19:15 (P=0.15) -0.79±0.80 (P>0.1)	A>T 24:13 (P=0.02) 1.18±0.67 (P<0.05)	C>G 27:7 (P=0.001) 3.58±1.12 (P<0.005)	C>G 25:12 (P=0.04) 1.44±0.93 (P<0.10)
EBV	60.1	T>A 23:21 (P=0.24) -2.18±1.64 (P>0.1)	T>A 49:37 (P=0.08) -1.49±1.10 (P>0.1)	G>C 30:14 (P=0.00007) -5.93±1.45 (P<0.0005)	C>G 64:22 (P<0.00001) 8.01±1.19 (P<0.005)
HSV	68.3	A>T 20:17 (P=0.15) 1.54±1.46 (P>0.1)	T>A 24:17 (P=0.037) -2.21±1.18 (P<0.05)	G>C 29:8 (P=0.0007) -4.61±0.98 (P<0.0005)	C>G 32:9 (P=0.00019) 3.21±0.88 (P<0.0005)

* Each sequence was examined using 1 kb windows moving in steps of 0.1 kb. Values for windows whose centres overlapped an ORF were averaged to obtain a value for that ORF. The first row of each dataset summarizes the relative proportions of the bases of each Watson-Crick base pair in the average ORF of each category (leftward or rightward transcribing). The second row shows the relative proportions of ORFs with positive and negative Chargaff differences, and the probabilities that the asymmetries in numbers of positives and negatives are not significant (calculated using the Wilcoxan signed ranks test).The third row shows average Chargaff differences for ORFs in each category (± standard error) with probabilities that the values are not significantly different from zero (t=test).

† HVS, *Herpes saimiri* (X64346);VZV, Varicella-Zoster virus (X04370); EBV, Epstein-Barr virus (V01555); HSV, *Herpes simplex* (X14112).

Figure 5-2. **Chargaff difference analysis of a section of the Epstein-Barr virus genome.** Open reading frames (ORFs) are shown as open boxes with arrows indicating transcription direction. C, G, A, and T are the number of bases counted in 1 kb windows which were moved along the sequence in 0.1 kb steps. Each data point corresponds to the middle of a window. Chargaff differences (%) are expressed as (C-G)/S (filled squares) where S = C+G, or as (A-T)/W (open circles) where W = A+T. The major ORF for Epstein-Barr nuclear antigen-1 (EBNA-1) is labelled.


125
100
75
50
25
0
-25
-50
(A-T)/W (%) & (C-G)/S (%)
EBNA-1
100
102
104
106
108
110
112
114
116
118
120
Position in Sequence (kilobases)

dramatically so. The latter gene is the *only* viral gene expressed in one type of EBV latency (the "EBNA-1 only program"; 216, 217). The ability of EBV to follow the normal rule, and hence "drive on the normal side of the road" may relate to the fact that the host's cytotoxic T cell response is not provoked during this latent state (218).

Table 5-3 shows Chargaff difference analysis of several other EBV latency-associated genes, which fall into three groups. EBNA-1 follows the normal rule. EBNAs 2-6 follow the opposite rule (the HTLV rule) with respect to the S bases, but not to the W bases. LMP1 and LMP2 unreservedly follow the HTLV-1 rule. These groupings correlate with some features of the biology of EBV (216, 217).

Impairing Host Defense Responses

Expression of a complementary antisense RNA can inhibit the expression of a cytosolic sense RNA (219). This would probably be initiated through loop-loop "kissing" interactions (200, 201). We report here that certain CG-rich viruses profoundly committed to latency strategies appear to invite interactions with host mRNAs, which would be initiated through complementary base pairing between loops. In vivo, C-rich loops of virus mRNAs would interact with G-rich loops of host mRNAs, (just as *in vitro* poly(rC) interacts *rapidly* at *low* temperatures with mRNA-synonymous DNA strands; 220). Thus, when triggered to move from the latent state to one of rapid productive cytolysis, the viruses would transcribe RNAs which, when released from the nucleus, would suddenly flood the cytosol with RNAs "driving on the wrong side of the road". The multiplicity of distracting loop-loop interactions may interfere with host cell "traffic" and therefore impair defense responses. This could be

Table 5-3. **Chargaff differences of latency associated genes of Epstein-Barr virus**

Transcription direction	Gene product	Chargaff differences (%) (A-T)/W	(C-G)/S	Rule followed*
Right	EBNA-1	A>T(38.8)	G>C (-38.1)	Normal, Normal
	EBNA-2	A>T (13.8)	C>G (31.4)	Normal, HTLV-1
	EBNA-3	A>T (4.6)	C>G (1.3)	Normal, HTLV-1
	EBNA-4	A>T (13.4)	C>G (3.0)	Normal, HTLV-1
	EBNA-5	A>T (1.9)	C>G (13.6)	Normal, HTLV-1
	EBNA-6	A>T (10.8)	C>G (1.9)	Normal, HTLV-1
	LMP-2†	T>A (-10.1)	C>G (2.5)	HTLV-1, HTLV-1
Left	LMP-1	A>T (6.8)	G>C (-23.6)	HTLV-1, HTLV-1

* Normally purines (R) are in excess when transcription is to the right, and pyrimidines (Y) are in excess when transcription is to the left. The opposite of the "normal rule" is the "HTLV-1 rule". Thus, for EBNAs 2-6 "Normal, HTLV-1" implies purine excess for the W bases, and pyrimidine excess for the S bases.

† Includes Exons 1, and 4 to 9; Exons 2 and 3 were omitted.

of adaptive value to the virus.

Messenger RNAs as "Antibodies"

So why does EBNA-1 mRNA follow the normal rule and "drive on the normal side of the road"? Distracted by the *messenger* role of mRNA molecules, we may fail to note that the diverse spectrum of cell mRNA species, like the diverse spectrum of antibodies in serum, constitutes a wide repertoire of specificities with the potential to react with complementary sections of non-self RNA "antigens". If EBNA-1 mRNA ("sense") in latent EBV-infected cells were not obeying the normal transcription direction rule and hence "driving on the normal side of the road" (to avoid "kissing" interactions with host mRNAs), then it is likely that a few host mRNAs would have a sufficient degree of complementarity ("antisense") to progress beyond the kissing interactions stage. Double-stranded RNA molecules of a length sufficient to alert host defense systems might then be formed. Interferon production is induced by double-stranded RNAs (203). Interferon would increase MHC protein expression (221). By obeying the normal rule EBNA-1 mRNA avoids interactions with host cell RNAs, and would thus fail to increase MHC expression in this way. This would impede the MHC-dependent cytotoxic T cell response, thus assisting to maintain the latent state.

Gly-Ala Region for Purine-Loading Non-Self RNA

It follows that the rightward transcribing gene encoding EBNA-1 should, where possible, have accepted mutations which increase its purine content (so that its mRNA would follow the normal rule). If this were not possible without disrupting protein functional

domains, the gene might have locally increased its content of purine-rich codons in inter-domain regions. Indeed, the EBNA-1 gene has a long "simple sequence" region (222) containing exclusively either glycine codons (GGN), or alanine codons (GCN). Table 5-4 shows that choices of third bases (N) in these codons are almost exclusively purines (223). Table 5-5 shows that inclusion of these purines serves greatly to increase Chargaff difference values in favour of the normal transcription direction rule.

Like those of other members of the herpes virus family, the EBV genome is very compact with little intergenic DNA; this suggests an evolutionary selection pressure to eliminate non-functional sequences. The remarkable prevalence of long simple sequences in EBV latency-associated genes has been explained by Karlin *et. al.* (222-224) in terms of adaptations at the protein level. Appearing to support this, Levitskaya *et al.* (218) presented evidence that the Gly-Ala region functions in cis at the protein level to inhibit antigen processing for MHC presentation. However, their evidence is also consistent with the Gly-Ala region being simply a device for purine-loading a foreign mRNA ("non-self") to make it appear like the host mRNA ("self"); this would outwit what might be a novel form of intracellular self/not-self discrimination (200, 204, 205). If selection had been acting at the protein level to conserve the Gly-Ala region there should not have been such extreme codon bias.

On the other hand, the bias might have arisen by the amplification of an initially small Gly-Ala-coding segment, which just happened to have the purine bias. However, in several other members of the herpes virus family there are similar purine-biases in long (>100 amino acids) simple sequence-encoding regions (Table 5-4). These might also be the result of

Table 5-4. **Codon usages of genes containing long simple sequences in Herpes simplex-related viruses**

Virus	Gene	Codons		Complete protein*	Less the simple sequence	Simple sequence alone	Human average† (%)
EBV	*BKRF1* (EBNA-1)	Gly	GGG	63	11	52	23
			GGA	144	43	101	27
			GGT	25	24	1	18
			GGC	19	19	0	33
		Ala	GCG	4	3	1	10
			GCA	85	2	83	24
			GCT	6	6	0	28
			GCC	8	8	0	39
HVS	*ORF 48*	Gly	GGG	34	2	32	23
			GGA	55	7	48	27
			GGT	1	1	0	18
			GGC	6	6	0	33
		Glu	GAG	92	10	82	55
			GAA	75	29	46	45
	ORF 73	Gly	GGG	2	2	0	23
			GGA	15	7	8	27
			GGT	1	1	0	18
			GGC	1	1	0	33
		Glu	GAG	2	1	1	55
			GAA	128	3	125	45
		Arg	AGG	2	2	0	48
			AGA	17	11	6	42
			CGG	1	1	0	34
			CGA	1	1	0	20
			CGT	4	3	1	15
			CGC	1	1	0	31
		Ala	GCG	2	2	0	10
			GCA	8	6	2	24
			GCT	43	6	37‡	28
			GCC	0	0	0	39
VZV	*ORF 11*	Gly	GGG	6	6	0	23
			GGA	28	16	12	27
			GGT	11	11	0	18
			GGC	5	5	0	33
		Glu	GAG	58	14	44	55
			GAA	30	30	0	45
		Asp	GAT	20	19	1	45
			GAC	35	16	19	55
		Ala	GCG	31	11	20	10
			GCA	19	19	0	24
			GCT	13	13	0	28
			GCC	13	13	0	39

* Values are the absolute number of codons in each protein segment.

† Data from 1657 human genes (226). Percentage distributions within each codon family are shown. For this purpose, the duplet and tetraplet arginine codons are considered separately.

‡ Pyrimidine-loading by virtue of this alanine codon is more than offset by the large excess of glutamate codons in *ORF 73*.

purine loading of mRNA. Of particular interest is *ORF 48* of HVS (T cell trophic), which is located in the HVS genome in a similar position to the EBNA-1-encoding gene in the genome of EBV (B cell trophic). Some of the ORFs are associated with CpG islands (Table 5-5): this is an expected feature of the promoter regions of latency-associated genes in viruses which have methylated CpGs (225).

Unlike EBV, HSV does not show CpG suppression (indicating no methylation of CpGs), and there are no long simple sequence regions. HSV would have pyrimidine-rich loops in most mRNAs (Table 5-2). Intriguingly, the main *Herpes simplex* RNAs transcribed during latency correspond to the "antisense" strand (227, 228). We predict that any part of these latency-associated transcripts which persist in the cytosol would be relatively purine-rich (229).

Charge Cluster Domains Decrease Immunogenicity of Other Domains

The use of simple sequence to purine-load mRNAs means that, at the protein level, the simple sequences often contain runs of charged amino acids (e.g. Glu, Asp). Karlin (224) refers to such regions as "hyper-charge runs", and notes that "for most of the hypercharge runs [in proteins] there is considerable variation in codon usage, which suggests an important function for these charge runs". However, our studies show that the variation is restricted to purine-rich codons (Table 5-4), implying selection acting at the nucleic acid level. This suggests that charge runs do not have an important function. When attempting to relate a protein's structure to function, we should consider the possibility that sometimes the function is that of the encoding nucleic acid, not of the protein itself (230). For example, to

Table 5-5. **Contribution of long simple sequences to Chargaff differences in ORFs of Herpes simplex-related viruses**

Virus	Gene	CpG island (local)[†]	Simple sequence length (codons)	Chargaff difference formula[*] (A-T)/W (%)			(C-G)/S (%)		
				Complete ORF	Less the Simple sequence	Simple sequence alone	Complete ORF	Less the simple sequence	Simple sequence alone
EBV	*BKRF1*	-	238	A>T (38.7)	A>T (17.2)	A>T (98.9)	G>C (-42.4)	G>C (-22.9)	G>C (-68.2)
HVS	*ORF 48*	-	301	A>T (22.8)	A>T (7.4)	A>T (63.0)	G>C (-55.6)	G>C (-13.5)	G>C (-93.7)
	ORF 73	-	183	A>T (46.1)	A>T (22.6)	A>T (75.5)	G>C (-26.7)	C>G (5.1)	G>C (-64.5)
VZV	*ORF 11*	+	102	A>T (2.1)	T>A (-3.8)	A>T (80.2)	G>C (-12.1)	G>C (-1.4)	G>C (-60.0)

[*] Parts of ORFs were considered as one window for calculating Chargaff differences (numbers in parenthesis).

[†] CpG islands are arbitrarily defined as >80 CpG dinucleotides/1 kb sequence window in a sequence which is generally depleted of CpG dinucleotides. The presence of a CpG island in regulatory regions suggests activity during latency. Absense of a local CpG island may mean that the promoter operating during latency is distant from the ORF. HSV is not generally CpG depleted, and has no ORF with a simple sequence >70 codons. Human herpes virus 6 (HHV6) has a 117 codon simple sequence in ORF *LJ1*, which displays purine loading with respect to the S bases, and has a CpG island.

compensate for a tendency of its protein product to provoke autoimmune attack by cytotoxic T cells, a gene might purine-load its mRNAs, thus generating long charge-rich alpha-helices which might be irrelevant to the function of the protein itself (231). In this respect, it is of interest to note the prevalence of charge clusters in antigens implicated in various autoimmune diseases. The clusters do *not* coincide with major autoantigenic epitopes (232). This suggests that charge cluster domains may not be the primary cause of the diseases, but may have evolved in response to the disease-provoking characteristics of other domains.

CHAPTER 6

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Are G0S genes responsive to Con-A independent of cycloheximide?

In Chapter 2, we were successful in demonstrating that *G0S2*, *G0S30/EGR1*, *G0S7/FOS* and *G0S3/FOSB* mRNA levels increase in response to the T cell polyclonal mitogen Con-A, independent of cycloheximide. This induction is consistent with the hypothesis that *G0S* gene products play a key role in the G_0/G_1 switch of T lymphocytes. Increased protein levels of G0S30/EGR1 (Chapter 3) and G0S3/FOSB (Chapter 4) were also noted in response to Con-A, lending further support to their putative role in T cell activation. Future studies should similarly assess the protein levels of other *G0S* genes in response to Con-A.

Do G0S gene mRNA levels increase rapidly after T cell activation?

In Chapter 2, it was determined that *G0S* gene mRNA levels increase very early after T cell activation. *G0S2* mRNA levels were found to increase in response to Con-A, peaking at 1-2 hr after addition and then declining. The other *G0S* genes examined, *G0S30/EGR1*, *G0S7/FOS*, and *G0S3/FOSB* displayed similar induction profiles in response to Con-A, peaking at 0.5 -1 hr, 0.5 hr and 1 hr after Con-A addition, respectively before declining. This early induction, during which time the G_0/G_1 switch is thought to occur, supports the notion that *G0S* genes encode key components of this cell cycle switch.

Are G0S genes responsive to other inducing agents involved in T cell activation?

It was also demonstrated that *G0S* gene mRNAs increase in response to the phorbol ester, TPA, and Ca^{2+} ionophore, ionomycin (Chapter 2). *G0S2* mRNA was found to increase significantly when TPA and ionomycin was added in combination to cultures. This not only supports the hypothesis that G0S2 plays a role in the G_0/G_1 switch, but also suggests that its induction is dependent on both PKC and Ca^{2+}-mediated pathways. A similar induction profile by TPA and ionomycin was noted for *G0S3/FOSB*. Alternatively, the induction of *G0S30/EGR1* and *G0S7/FOS* was found to be primarily due to TPA and to a lesser extent due to ionomycin. Although this supports the notion that G0S30/EGR1 and G0S7/FOS play important roles in the G_0/G_1 switch, it suggests that their induction is primarily dependent on a PKC-mediated pathway.

Can the induction of G0S genes be blocked by CsA?

It was found that the early expression of *G0S2* mRNA, in response to Con-A or TPA + ionomycin, is CsA-sensitive (Chapter 2). This was not found for the other immediate-early genes, *G0S30/EGR1*, *G0S7/FOS*, and *G0S3/FOSB*. This CsA-sensitivity of *G0S2* mRNA expression suggests that G0S2 plays a critical role in the commitment of cells to enter the G_1 phase of the cell cycle. This finding also suggests that early inhibition of *G0S2* mRNA by CsA may be important for the immunosuppressive action of CsA. Our findings raise the possibility of using *G0S2* as a reporter gene in future studies to study CsA action. Noting that these studies have focussed on the kinetics of mRNA expression, future studies should investigate whether these affects are also noted at the protein level. Future work should also

examine the ability of recombinant G0S2 addition to CsA-treated cultures to re-establish T cell activation.

Although putative NFAT and AP1 sites have been identified in the promoter region of *G0S2* (Chapter 2), studies are required to determine if the transcriptional regulators, NFAT and AP1 actually bind the *G0S2* promoter, resulting in transactivation. Future work should include co-transfection assays with the 5' flanking region of *G0S2* fused to a chloramphenicol acetyltransferase (CAT) reporter gene to address this question.

Much work in the field of transplantation medicine has involved the isolation and characterization of CsA analogues as well as other immunosuppressants. This work has focussed on identifying more potent immunosuppressants that are less nephrotoxic than CsA (67). Future work should also involve the use of these other immunosuppressants, such as FK506 and rapamycin. We suspect that *G0S2* expression will also be sensitive to FK506, which is thought to block similar pathways as CsA. Conversely, we predict that rapamycin, which is thought to act at a stage after IL2 synthesis, will not effect *G0S2* expression. Moreover, in continuing to assess the role of *G0S* genes in T cell activation, future studies should also examine the CsA-, FK506- and rapamycin-sensitivity of other *G0S* genes, such as *G0S8/RGS2*, whose expression is Ca^{2+}-dependent (35).

Attempting to express recombinant G0S2 protein

At present, lack of an anti-G0S2 antibody and recombinant G0S2 protein has precluded us from carrying out the above mentioned studies. To this end, the expression of recombinant G0S2 in *E. coli* was undertaken, yet it proved unsuccessful (See Appendix A1).

As G0S2 is enriched in human codons that are infrequently used in *E. coli* (27), future studies should include re-engineering the *G0S2* cDNA sequence to maximize usage of codons frequently used by *E. coli*. This may help to facilitate G0S2 protein expression. The presence of a hydrophobic region, particularly the transmembrane segment of G0S2 (Chapter 2), may have also contributed to the lack of G0S2 protein expression. Such regions often exhibit a toxic effect on host cells, likely due to an association of the protein with crucial membrane systems (151). The removal of this region may also facilitate G0S2 protein expression in future studies. The inability to express G0S2 protein may also reflect the instability of G0S2 in *E. coli*. This is generally found with short protein sequences that are quickly degraded by cellular proteases. Expression of recombinant G0S2 in eukaryotic expression systems (baculovirus or yeast), may also facilitate protein expression. After the expression and purification of recombinant G0S2, work should then focus on developing a rabbit affinity-purified polyclonal anti-G0S2 antibody.

Does inhibiting G0S gene expression by antisense oligonucleotides inhibit downstream events?

In our studies to inhibit the expression of *G0S30/EGR1* and assess the effects on downstream events, inhibition of lymphocyte activation (as measured by ^{3}H-uridine incorporation into RNA) was noted using a low concentration of a *G0S30/EGR1*-antisense oligonucleotide (Chapter 3). However, this was not found to correlate with a decrease in either mRNA or protein levels of *G0S30/EGR1* (Chapter 3). This raises the possibility that we may be targeting another gene that is important for T cell activation. Future studies

employing ddRT-PCR. using the *G0S30/EGR1*-complementary oligonucleotide as an upstream primer rather than the commonly used 10 nucleotide random primer. may help to identify the gene(s) that is targeted by this oligonucleotide. Another possible explanation for this finding is that there is a constitutive basal level of G0S30/EGR1 protein found in the cytoplasm which may be either non-functional or serve an alternate role than its nuclear transcription factor counterpart. Activation of T lymphocytes results in transient induction of *G0S30/EGR1* mRNA and protein which is sensitive to the antisense oligonucleotide employed. However, when G0S30/EGR1 protein levels are assayed there is no change in concentration noted due to the overwhelming presence of the "non-functional" consitutive G0S30/EGR1. Future studies should address this hypothesis using immunohistochemistry to assess the presence of G0S30/EGR1 in the cytoplasm and nucleus before and after T cell activation.

Although we were not successful in assessing the role of G0S30/EGR1 in cell cycle progression, this work has advanced our current knowledge of the use of antisense oligonucleotides in the inhibition of target gene expression. Our studies of G0S30/EGR1 suggest that cells have an endogenous defense system protecting them from foreign oligonucleotides that can be activated above a "critical" concentration. Our finding that very low concentrations of an antisense oligonucleotide can inhibit lymphocyte activation suggests that it may not be necessary to bombard cells with extremely high concentrations of oligonucleotides in order to inhibit target gene expression. Furthermore, our findings suggest that it may not be necessary to use artificial means to facilitate entry of oligonucleotides into cells. Thus, our findings have important implications to the use of

antisense oligonucleotides as therapeutic agents.

Although our inhibition studies of *G0S3/FOSB* expression were equally unsuccessful in determining its role in cell cycle progression, they do shed some light on the importance of oligonucleotide concentration. RNA synthesis was found to be inhibited with low oligonucleotide concentrations in control (no Con-A), but not in Con-A-stimulated cultures (Chapter 4). Our findings suggest that oligonucleotide concentrations required to effectively inhibit the low basal expression of *G0S3/FOSB* were identified. However, those concentrations required to inhibit the expression of *G0S3/FOSB* in Con-A-induced samples were not identified. Together with the results of the *G0S30/EGR1* antisense study, our work emphasizes the importance of identifying an oligonucleotide concentration that is both *sufficient* to exert its inhibitory action and yet will not be detected by putative host cell defense systems.

In retrospect, in attempting to inhibit the expression of *G0S30/EGR1*, our initial assay of [^{3}H]uridine incorporation, rather than G0S30/EGR1 protein levels, may have caused us to overlook the critical inhibitory oligonucleotide concentration. In other words, we may have actually inhibited the expression of *G0S30/EGR1*, which in-turn, did not affect [^{3}H]uridine incorporation. Perhaps expression of another gene was up-regulated as a result of inhibiting *G0S30/EGR1* expression, thus serving a compensatory function. Therefore, future studies should also examine the effects of varying antisense oligonucleotide concentrations on G0S30/EGR1 protein levels.

Future studies assessing the role of G0S3/FOSB should also explore higher concentrations of antisense oligonucleotides, varying preincubation times as well as harvest

times. A transgenic mouse study in which FOSB was 'knocked out' demonstrated a down-regulation of two potential AP-1 targets, stromelysin and collagenase (197). Future inhibition studies of *G0S3/FOSB* expression by antisense oligonucleotides followed by RT-PCR and immunoblot analyses would assess whether this downregulation also occurs in human T lymphocytes.

Although localization studies have been carried out for high oligonucleotide concentrations, the same is not true for the relatively low concentrations that we have found to inhibit lymphocyte activation (77). Such experiments using a fluorescein isothiocyanate-labelled oligonucleotide would have assessed whether the oligonucleotide was taken up by the T cells and would have also elucidated the intracellular localization of these oligonucleotides. However, financial considerations precluded such experiments. Therefore, future work should include localization studies to determine where oligonucleotides at these low concentrations are found. Moreover, chemical cross-linking studies using a biotin-labelled oligonucleotide may also help to identify the potential protein targets of oligonucleotides, such as cell surface receptors, which could be involved in entry of oligonucleotides at these low concentrations into the cell.

In order to further test the "stealth" hypothesis, more studies should be carried out on other target genes. These targets should not only include other *G0S* genes, such as the regulator of G-protein signalling, *G0S8/RGS2*, but also non-lymphoid genes. This would evaluate whether the observation that relatively low concentrations of antisense oligonucleotides are effective at inhibiting gene expression, potentially by circumventing a putative endogenous defense system, is specific to human blood mononuclear cells or is also

true of other cells.

Future studies should involve ddRT-PCR to identify any gene(s), whose expression is up- and/or down-regulated as a result of inhibiting *G0S* gene products using antisense oligonucleotides. This may help to identify genes whose products can functionally compensate for G0S proteins as well as those that are potential "downstream" targets of these proteins.

Although previous studies have investigated a potential correlation between the extent of inhibition and RNA secondary structure, results were inconsistent (79, 81). The results of our bioinformatic analysis that tested several energy minimization values (FONS, FORS-M, FORS-D; See Chapter 4) were equally inconclusive. Using the one consistent trend noted in our bioinformatic analysis as a guide (Chapter 4), antisense oligonucleotides targeted to regions of *FOSB/G0S3* mRNA with high (ASCH) and low (ASCL) base composition-determined stem-loop potential were designed. However, the results of this inhibition study also proved inconclusive.

Are purine-rich RNA loop domains prevalent in retroviruses and viruses committed to latency?

The inconsistent findings of our antisense oligonucleotide prediction studies (Chapter 4) may reflect the fact that our knowledge of factors that influence RNA structure is incomplete. To this end, a computational (Chargaff difference) analysis of several viral genomes was carried out. Previous studies have shown that many organisms have purine-rich clusters in mRNA that correspond to the loop domains of potential stem-loop structures

(199). In Chapter 5 this purine-richness was also noted in the retroviruses, SFV-1, HIV-1 and RSV. Conversely, HTLV-1, a retrovirus profoundly committed to latency exhibited pyrimidine-richness in its RNA. In order to resolve this paradox between HTLV-1 and the other retroviruses, members of the herpes virus family, also committed to latency, were examined. A similar pyrimidine-richness was noted in the majority of transcripts of EBV with the exception of EBNA-1, which was found to be purine-rich. Our Chargaff difference analysis suggests that EBV may resort to "purine-loading" *EBNA-1* in order to allow this latency-associated transcript to avoid interactions with host cell mRNAs, interferon induction and MHC production. Future studies should examine the frequency of purine-loading in latency-associated transcripts of other latent viruses. This knowledge of frequently occuring purine-rich loops of mRNA stem-loop structures (exception are viruses committed to latency) may help to design more effective antisense oligonucleotides. Future inhibition studies should design purine-rich antisense oligonucleotides targeted to the rarely occurring pyrimidine-rich loops of target mRNA stem-loop structures. In this way one may be able to avoid interactions between antisense oligonucleotides and distracting purine-rich loops which are very common. In future, identification of a consistent predictor of effective antisense oligonucleotides may require consideration of not only RNA secondary structure, but also tertiary structure. In this way, one could potentially identify stem-loops that are accessible to incoming oligonucleotides and avoid those that may be hidden and inaccessible.

SUMMARY AND CONCLUSIONS

This work has described the characterization of putative G_0/G_1 switch genes, *G0S2*, *G0S30/EGR1*, *G0S7/FOS* and *G0S3/FOSB* in order to help establish the roles of *G0S* gene-encoded products in this cell cycle switch. To this end, the following questions were addressed: (1) Are *G0S* genes responsive to Con-A independent of cycloheximide? (2) Do *G0S* gene mRNA levels increase rapidly after T cell activation? (3) Are *G0S* genes responsive to other inducing agents involved in T cell activation? (4) Can the induction of *G0S* genes be blocked by CsA? (5) Does inhibiting *G0S* gene expression by antisense oligonucleotides inhibit downstream events? The need for a consistent predictor of effective antisense oligonucleotides led us to investigate a possible correlation between some aspect of RNA secondary structure and antisense oligonucleotide inhibitory efficacy. To further our understanding of factors that influence RNA structure, which in-turn could help to design more effective antisense oligonucleotides, the following question was also addressed: (6) Are purine-rich RNA loop domains prevalent in retroviruses and viruses committed to latency?

Our studies were successful in having revealed the early expression of *G0S2*, *G0S30/EGR1*, *G0S7/FOS* and *G0S3/FOSB* mRNA in response to Con-A, TPA and ionomycin. This work also clearly demonstrated that early mRNA expression of *G0S2* is CsA-sensitive (Chapter 2). These findings support the hypothesis that *G0S* gene products play key roles in the G_0/G_1 switch.

Although our antisense oligonucleotide inhibition studies of *G0S30/EGR1* and *G0S3/FOSB* expression were not successful in assessing their hypothesized roles in cell cycle progression, the importance of oligonucleotide *concentration* required to achieve inhibition

is evident. [^{3}H]Uridine incorporation studies using an antisense oligonucleotide complementary to *G0S30/EGR1*, suggest that cells possess an endogenous defense system protecting them from foreign oligonucleotides, that may be "triggered" above a critical concentration (Chapter 3). The inhibition studies of *G0S3/FOSB* that followed (Chapter 4) provided further support to the importance of oligonucleotide concentration. The results of this work suggest that basal mRNA levels of *G0S3/FOSB* are so low in control cultures that we can inhibit protein expression of *G0S3/FOSB* and the downstream event of RNA synthesis using low concentrations of antisense oligonucleotides. However, this inhibition cannot be achieved using the same concentrations in Con-A-stimulated cultures when *G0S3/FOSB* mRNA levels are transiently elevated. Therefore, in order to inhibit the expression of target genes it is imperative to identify an antisense oligonucleotide concentration that is both sufficient to exert its inhibitory effect and yet will not be detected by host cells.

Other factors that may optimize the inhibition of target gene expression were explored. To this end, a search for a possible relationship between antisense oligonucleotide-mediated inhibition and some aspect of RNA secondary structure was carried out. The findings of our bioinformatic analysis were inconclusive. One consistent trend was found between the extent of inhibition and *low* base composition-determined stem-loop potential. However, the results of our inhibition studies using candidate antisense oligonucleotides targeted to *G0S3/FOSB* contradicted these predictions.

Recognizing that these results may have been a reflection of our incomplete understanding of RNA structure, a Chargaff difference analysis of retroviruses and herpes

viruses was carried out. Our findings draw attention to the phenomenon of "purine-loading" of mRNA which may be used to design more effective antisense oligonucleotides (Chapter 5).

The findings outlined in this thesis regarding the mRNA expression and CsA-sensitivity of *G0S* genes lend further support to the role of *G0S* gene products in the G_0/G_1 switch of T lymphocytes. The usefulness of antisense oligonucleotides are not immediately clear in the inhibition studies of *G0S* genes presented in this thesis. These findings may reflect the many uncertainties that still plague this technology. However, investigations such as those reported in this thesis will further our understanding of this invaluable molecular tool and will allow us to optimize its use as both a biological and therapeutic agent.

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APPENDIX A1

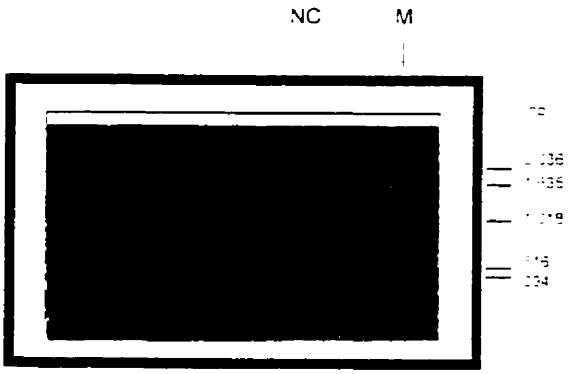
This section describes the successful cloning strategy used to clone *G0S2* cDNA into expression vector pET19b. It also describes attempts made at expressing G0S2 protein in *E. coli* which were unsuccessful.

Cloning of G0S2 cDNA into pET19b

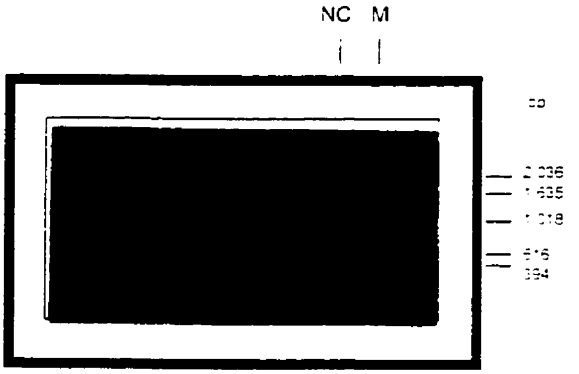
Using full length *G0S2* cDNA in pBR322 , two primers were designed in order to generate a 549 bp PCR product having an *NdeI* site at the 5' end, and a *BamHI* site at the 3' end. The primers were: 5'-TCTAGAATTCATATGGAAACGGTCCAGGA-3' (upstream) and 5'-TGAGCTCGAGGATCCCAAACTCCTTTGGT-3' (downstream). These primers were also designed so that each end of the resultant PCR product would have an additional 9 bp so as to facilitate subsequent restriction enzyme digestion by *NdeI* and *BamHI*. Verification that the desired PCR product was generated was carried out using agarose gel electrophoresis followed by ethidium bromide staining (Fig. A1-1A). Restriction enzyme digestion of pET19b (Novagen, Madison) was carried out using *NdeI* and *BamHI*. The *NdeI/BamHI* digested *G0S2* cDNA (PCR product) was then cloned into the *NdeI/BamHI* digested pET19b vector using *E. coli* JM83 cells as the host strain. After colony screening, PCR amplification and agarose gel electrophoresis (Fig. A1-1B) verified that the cloning had been successful. Transfection of pET19b + *G0S2* cDNA into the *E. coli* λDE3 lysogen of strain BL21, used as the host for G0S2 expression, was confirmed by PCR screening of 10 single colonies (Fig. A1-1C). DNA sequencing analysis (Cortec DNA Service Laboratories

Figure. A1-1. **Verificaton of successful cloning of *G0S2* cDNA into pET19b**. Agarose gel electrophoresis (1%) and ethidium bromide staining was carried out after PCR amplification of (A) *G0S2* cDNA in pBR322, (B) picked colonies following the cloning of *NdeI/BamHI* digested *G0S2* cDNA into *NdeI/BamHI* digested pET19b, and (C) picked colonies following the transfection of pET19b + *G0S2* cDNA into BL21 cells. NC refers to the negative control sample in which no template was added to the PCR sample mixture and M refers to 1 kb ladder markers used.

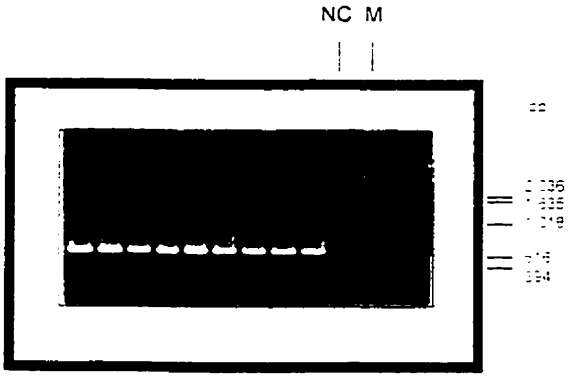
(A)



(B)



(C)



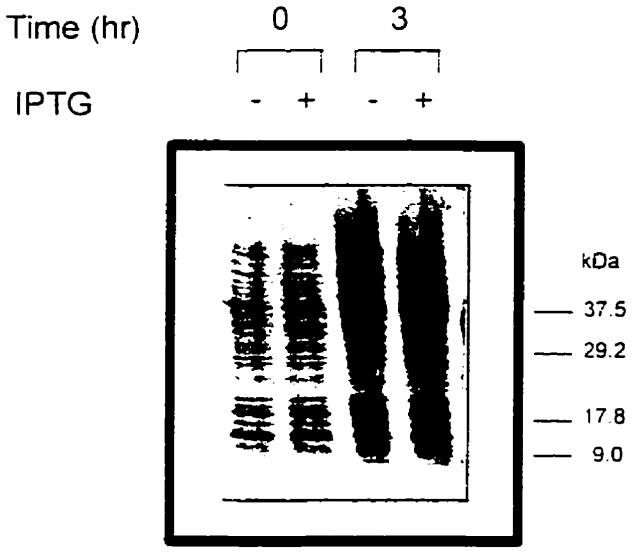
Inc.) that was carried out not only verified that cloning had been successful, but also confirmed that the T7 promoter and lac operator regions were intact and had not been disrupted during the cloning procedure.

Bacterial Expression and Purification of G0S2 protein

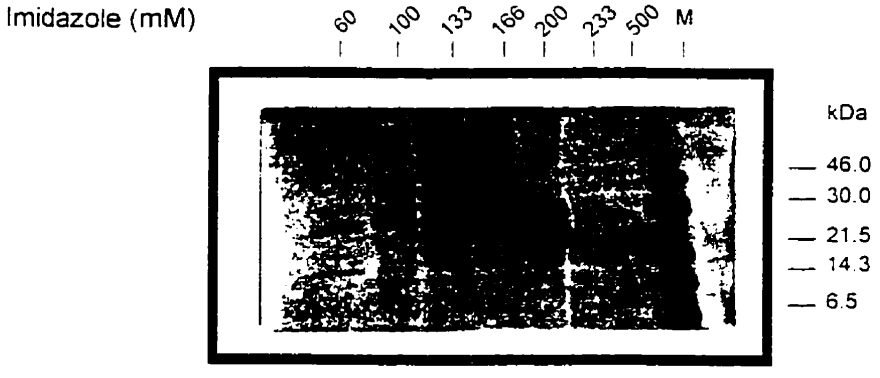
Target gene expression and rapid screening of cultures were carried out as outlined by the Novagen pET System Manual. Fig. A1-2 shows the results of our expression and purification as analyzed by SDS-PAGE and coomassie blue staining. There was not found to be increased intensity of any one particular band in the expected 12.5 kDa region in the +IPTG lane relative to the -IPTG lane (Fig. A1-2A). Our inability to note a G0S2 band in Fig. A1-2A may simply have been a function of weak G0S2 protein expression as well as high levels of endogenous *E. coli* contaminating proteins. We therefore attempted to purify G0S2 protein, assuming that it had been expressed. Purification was carried out by exploiting the 10 consecutive histidine residues (His•Tag sequence) at the N-terminal end of the target protein which can bind to divalent cations (Ni^{2+}) immobilized on the His•bind™ metal chelation resin. Briefly, cell extracts were prepared from samples that had been treated for 3 hr with 1 mM IPTG and were loaded onto a column containing His•bind resin. Elution of the target protein was carried using increasing concentrations of imidazole, however as shown by Fig. A1-2B, the only bands noted were greater than the expected 12.5 kDa of G0S2. Moreover, these bands that were noted were eluted at very low imidazole concentrations suggesting that they were only loosely associated with the resin and could

Figure A1-2. **Inability to express G0S2 protein in *E. coli*.** (A) SDS-PAGE (15%) analysis of bacterial cultures at time 0 hr and 3 hr following IPTG (1 mM) treatment. (B) SDS-PAGE (15%) analysis of samples eluted from the His•bind™ metal chelation resin using increasing concentrations of imidazole.

(A)

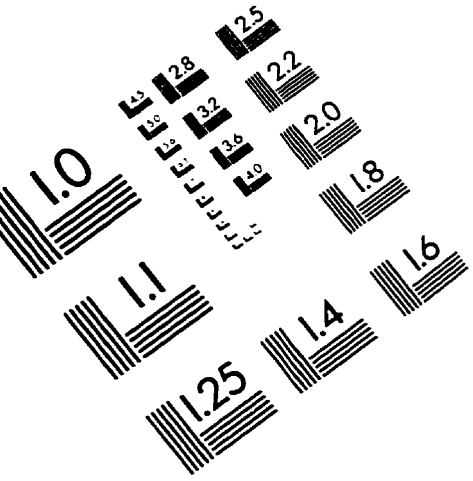

Time (hr)
0
3
IPTG
- + - +
kDa
37.5
29.2
17.8
9.0

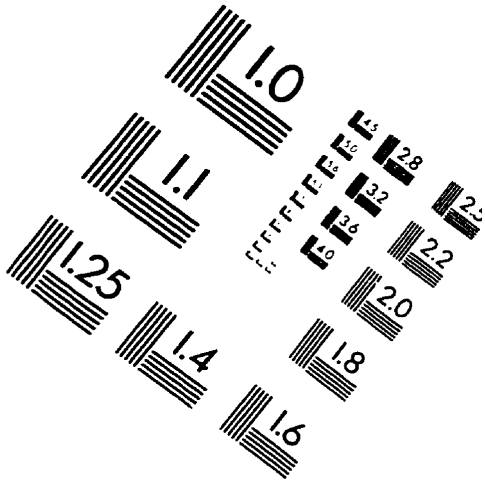
(B)

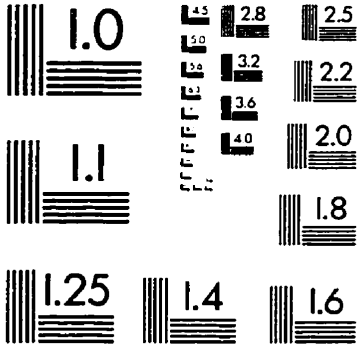

Imidazole (mM)
60
100
133
166
200
233
500
M
kDa
46.0
30.0
21.5
14.3
6.5

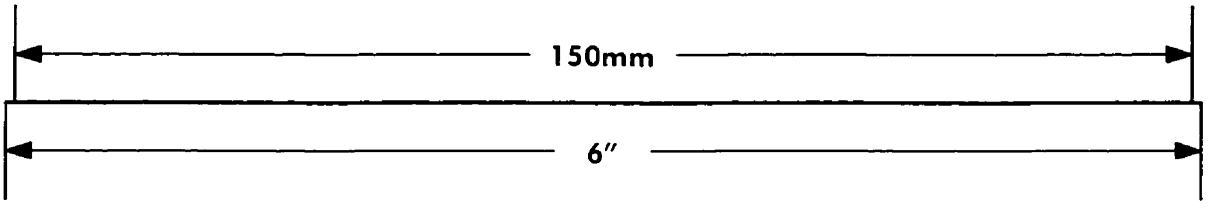
therefore represent endogenous *E. coli* proteins.

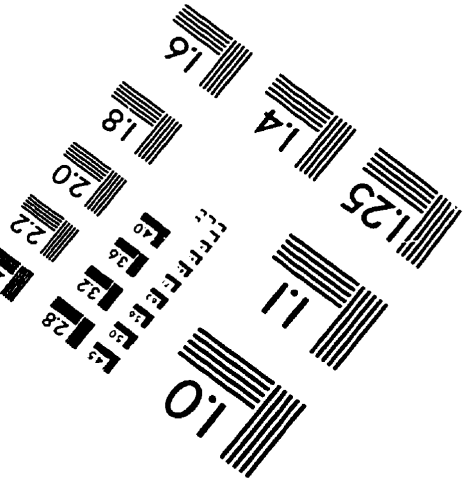
IMAGE EVALUATION TEST TARGET (QA-3)

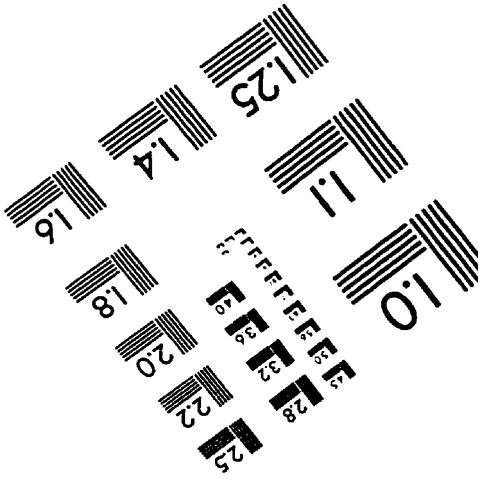












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