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**Effect of GM-CSF on RNA and Protein
in Human Peripheral Blood Granulocytes**

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Thesis submitted to the Department of Biochemistry, Microbiology and Immunology in
partial fulfillment of the requirements for the degree of Doctor of Philosophy

University of Ottawa

Ottawa, Ontario, Canada

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ABSTRACT

Human granulocytes are the most abundant circulating leukocyte, and are critical in protection against microbial infection. These cells are terminally differentiated and highly specialized, possessing very little RNA and protein synthesis. It was believed that transcription and translation were unnecessary for granulocyte functioning, but it is now understood that these processes play a role in some responses. The cytokine GM-CSF, which can be released at inflammatory sites, is an important granulocyte effector. At least one GM-CSF-induced response (i.e., delay in spontaneous apoptosis) has been shown previously to require both transcription and translation.

I undertook to examine RNA and protein in these cells, given the importance and need of study. (i) Using DDRT-PCR, *hsgk* (a putative serine/threonine kinase) mRNA was found to be upregulated ~10-fold for at least 12 h with ≥ 0.1 ng/mL GM-CSF. (ii) GM-CSF increased [^3H]uridine uptake into RNA by ~10-fold in 6-h cultures. At least 90% of ^3H -RNA was derived from polymerase II, but most appeared to be nonpolyadenylated hnRNA. Granulocytes appear sluggish to synthesize mature cytoplasmic mRNA. Probing granulocyte total RNA with serglycin proteoglycan intron sequences revealed that transcriptional elongation may be the rate-limiting step. (iii) An unknown 26-27 kDa protein demonstrated the largest increase in [^{35}S]methionine-labeling (2-4 fold) with GM-CSF-stimulation. This protein could not be identified. (iv) Immunoblotting of Bcl-2 family members revealed only the pro-apoptotic Bax. These proteins apparently are not involved in GM-CSF-induced apoptotic delay. (v) hTegt mRNA was found highly expressed in granulocytes, but was unaffected by GM-CSF. This conserved, little-studied protein was analyzed further. Although the function of hTegt in granulocytes and other cells could not be determined, several properties were uncovered that could be beneficial in future studies. More RNA and protein species remain to be identified in granulocytes, but this work has furthered understanding of the few that are expressed.

-II-

To my mother

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LIST OF ABBREVIATIONS

AIF	apoptosis-inducing factor
AML	acute myeloid leukemia
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BGH	bovine growth hormone
BPI	bactericidal/permeability-increasing protein
CAP	cationic antimicrobial proteins
Caps	3-(cyclohexylamino)-1-propanesulfonic acid
CDTA	trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid
CFU-G	granulocyte colony forming unit
CFU-GEMM	granulocyte-erythrocyte-macrophage-megakaryocyte colony forming unit
CFU-GM	granulocyte-macrophage colony forming unit
CGD	Chronic Granulomatous Disease
CIP	calf intestinal alkaline phosphatase
CMV	cytomegalovirus
CR _x	complement receptor "x"
CT	1 mM CDTA, 10 mM Tris/HCl (pH 7.5)
C5a	complement factor 5a
DDRT-PCR	differential display reverse transcription PCR
DEPC	diethyl pyrocarbonate
DHFR	dihydrofolate reductase
DIC	differential interference contrast microscopy
DMEM-HG	DMEM cell culture media containing high glucose
DMSO	dimethyl sulfoxide
DTT	dithiothreitol

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ER	endoplasmic reticulum
EST	expressed sequence tag
FCS	fetal calf serum
FLAP	5-lipoxygenase-activating protein
fMLP	<i>N</i> -formyl-Met-Leu-Phe
G-CSF	granulocyte colony stimulating factor
Glc	glucose
GM-CSF	granulocyte-macrophage colony stimulating factor
Gst	glutathione S-transferase
HA	hemagglutinin
<i>hsgk</i>	human serum- and glucocorticoid-regulated kinase
<i>hsgly</i>	human serglycin
Hsp	heat shock protein
hTegt	human testis-enhanced gene transcript
hTS	human thymidylate synthase
IEF	isoelectric focusing
IFN	interferon
IL-x	interleukin-"x"
IL-1Ra	interleukin-1 receptor antagonist
IPTG	isopropyl- β -D-thiogalactopyranoside
LPS	lipopolysaccharide
mw	molecular weight
NBT	nitro blue tetrazolium
NCBI BLAST	National Center for Biotechnology Information, Basic Local Alignment Search Tool
NGAL	neutrophil gelatinase-associated lipocalin

NP-40	Nonidet P40
NLS	nuclear localization signal
ORF	open reading frame
PAF	platelet activating factor
PEI	polyethyleneimine
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PMN	polymorphonuclear granulocytes
Poly(P)	linear inorganic polyphosphates
p27gran	unknown 26-27 kDa granulocyte protein
RA	retinoic acid
ROS	radical oxygen species
RT	reverse transcription
SD	standard deviation
SEM	standard error of the mean
TBST	0.15 M NaCl, 0.1% (v/v) Tween 20, 10 mM Tris/HCl (pH 8.0)
TCA	trichloroacetic acid
TNF	tumor necrosis factor
UTR	untranslated region
v-ATPase	vacuolar H ⁺ -ATPase
10×SSC	1.5 M NaCl, 0.15 M sodium citrate/HCl (pH 6)

Chapter 1

General Introduction

1.1 - Types of polymorphonuclear granulocytes

Polymorphonuclear granulocytes (PMNs) are nondividing leukocytes that, as their name suggests, contain multisegmented nuclei and numerous cytoplasmic granules. Mature PMNs are composed of three subtypes: neutrophils (90%), eosinophils (4-5%) and basophils (1%). The remaining 4-5% of PMNs in the blood are immature band neutrophils [1]. The names of the granulocyte subtypes were coined based on their pattern of staining with Wright's stain (a mixture of methylene blue, oxidized methylene blue and eosin). The granules of the basophil contain a net anionic charge (e.g., from heparin) and stain deep blue-black with basic dyes. The granules of eosinophils contain cationic compounds and stain red with eosin. The granules of human neutrophils stain neutrally as a purplish colour, although this is not necessarily true of neutrophils from all organisms [1]. Human neutrophils comprise 61% ($\bar{x}$, $n = 1002$) of the total circulating blood leukocytes [2]. As will be discussed later, neutrophils are critical in defending against acute bacterial and fungal infections. Less is known about the normal functions of eosinophils and basophils, although they have been implicated in allergic reactions and in defence against parasites. Eosinophils and basophils will not be discussed in this thesis; more detailed reviews can be found elsewhere [3-5]. This thesis will deal with neutrophilic PMNs, which represent the majority of granulocytic cells.

1.2 - Comparison of peripheral blood neutrophils to cultured hematopoietic tumour cells

One of the best studied neutrophil-like tumour cells is the differentiated HL-60 cell. HL-60 were derived from acute promyelocytic leukemia cells and are considered to be

immortalized neutrophil precursors [6]. (Neutrophil differentiation occurs via the following scheme: pluripotent stem cell → CFU-GEMM → CFU-GM → CFU-G → myeloblast → promyelocyte → myelocyte → metamyelocyte → band or stab cell → mature neutrophil [7,8]). HL-60 can be differentiated to neutrophil-like cells by culturing *in vitro* with many chemical agents, but the most widely used differentiating agents are all *trans*-RA and DMSO [9,10]. HL-60 cells are more easily manipulated than peripheral blood granulocytes, they can provide more consistent results (i.e., there is no donor variability), and they do not require a bank of blood donors. Differentiated HL-60 do possess certain characteristics of mature neutrophils such as segmented nuclei, primary granules, complement receptors, phagocytic capabilities, chemotactic responses, the ability to reduce NBT, cessation of proliferation and eventual apoptotic cell death [9,11]. However, it must be emphasized that these cells are incompletely differentiated and only approximate the properties of normal peripheral blood neutrophils. Differences between the two cell-types include the following. (i) Differentiated HL-60 have no lactoferrin and no alkaline phosphatase, suggesting the absence of secondary granules and phosphasomes [12,13]. (ii) Differentiated HL-60 express very little *c-fos* mRNA compared to peripheral blood granulocytes [14,15]. (iii) Although p53 cannot be detected in circulating neutrophils [16], p53 levels have been shown to rise during normal myeloid differentiation in bone marrow [17]. HL-60 cells have deleted p53 alleles and cannot express the protein [18]. (iv) Differentiation of HL-60 cells downregulates both Bcl-2 and Bax protein [19], while Bax levels are high in peripheral blood neutrophils [20]. (v) Our lab has found detectable levels of hTS protein in differentiated HL-60 cells but not in mature neutrophils [21]. Thus, there are many differences between these two cell-types as well as

many similarities.

For most of the work in this thesis, human neutrophils that had been isolated from peripheral blood were utilized; differentiated HL-60 cells were used only occasionally for comparison. Neutrophils require daily isolation from the blood of volunteers. Neutrophils also contain potent ribonucleases and very little RNA ($\sim 0.2 \mu\text{g}$ per 10^6 cells), properties that can hinder RNA isolation. Despite these difficulties, I developed methodologies to allow the identification of novel RNA and protein species in these cells. The significance of transcription and translation in the functioning of neutrophils will be discussed in more detail in sections 1.5 and 1.8. Neutrophils are, however, unsuitable for transfection of protein expression constructs because of their short lifespan and low rates of RNA and protein synthesis. This shortcoming was insurmountable. Therefore, I had to use other cultured human cells when transfections were performed as described in Chapter 6.

1.3 - Neutrophil morphology and biochemistry

Mature human neutrophils found in the peripheral blood are spherical with a diameter of 10-15 μm and an average volume of 346 fL [22]. Twenty-one percent of this volume is occupied by the nucleus [23]. Neutrophil nuclei are heterochromatic with chromatin compaction occurring at the nuclear periphery. In chicken PMNs, this chromatin compaction is believed to be mediated by a protein called MENT, which acts as a "glue" within and between chains of nucleosomes [24]. The high content of heterochromatin suggests that a large amount of the genome is transcriptionally inactive. Nuclei have an unusual multisegmented structure. Adjacent segments are interconnected by thin chromatin filaments

that can be difficult to discern under light microscopy. Although the number of segments varies, the mode is 3 segments [25]. From his pioneering work, Elie Metchnikoff believed that the segments enabled neutrophils to squeeze through tissue more easily during intravasation [26]. However, neutrophils from individuals with congenital Pelger-Huet anomaly lack typical nuclear segmentation yet demonstrate no functional defect [27]. The purpose of nuclear segments and how they are formed remains unclear. Under phase-contrast microscopy, segments can appear grouped around the centrosome in the centre of the cell [28]. The role that this organelle plays in segmentation, if any, has not been addressed.

Mature neutrophils contain numerous membrane-enclosed granules that occupy 15.4% of the cell volume [23]. Granules can be divided into 4 classes, namely azurophil (primary) granules, specific (secondary) granules, gelatinase (tertiary) granules and secretory vesicles (phosphasomes) [7,29,30]. These granule classes vary in their size, density, content and time of biosynthesis. It should be noted that heterogeneity does exist within the classes. For example, azurophil granules can exist as nucleated (crystal containing) structures, as large structures with weak peroxidase staining and as small, electron dense structures with strong peroxidase staining [7]. Only the azurophilic granules are large enough and stain darkly enough to be seen as distinct organelles under light microscopy. Azurophilic granules are considered lysosomal in origin due to their content of acid hydrolases and mannose 6-phosphate-containing glycoproteins. However, Cieutat *et al.* failed to find lysosome-associated membrane proteins (LAMP) in azurophilic granules [31]. Therefore, by current criteria, azurophilic granules cannot be considered lysosomes. Phosphasomes are formed

in band cells and segmented neutrophils and have been shown to contain ROS [32-34]. Phosphasomes translocate completely to the plasma membrane with nanomolar concentrations of fMLP, while azurophilic granules are released only to a minor extent [32,35]. There is an inverse correlation between granule mass and the ease at which they are exocytosed; granules that are formed early in neutrophil maturation (i.e., larger and denser granules), are usually more difficult to mobilize [7,30]. In general, the contents of granules enable a neutrophil to carry out its microbicidal functions. Therefore, many granule constituents are hydrolytic enzymes (e.g., lysozyme) or antimicrobial proteins (e.g., defensins [36,37]). The properties of the four granule subtypes are summarized in Table 1.I.

Mature neutrophils possess unusual morphological features such as segmented nuclei and specialized cytoplasmic granules, but they lack other common subcellular structures. In electron micrographs, free ribosomes, ER and Golgi are scarce, suggesting severely reduced activity of these structures. Nucleoli are not visible [1,25,38]. Also, relatively few mitochondria are present (20-30 per cell, 0.6% of cell volume) [23,39] compared to other cells (e.g., 500-2500 per rat hepatocyte, $18.4 \pm 2.2\%$ of cell volume) [40,41]. In a resting neutrophil, most ATP is synthesized via anaerobic glycolysis using extracellular glucose as an energy source [1]. Hexose monophosphate shunt activity is low in the resting cell, representing ~1-2% of glucose metabolism [44]. Glycogen granules are readily visible by electron microscopy; neutrophils contain the highest glycogen content of all blood leukocytes [45]. Glycogen levels remain fairly constant in the resting cell but drop during phagocytosis due to increased glycogenolytic activity [1]. Also during phagocytosis, neutrophilic hexose monophosphate shunt activity can account for as much as 30% of

Table 1.I
Summary of neutrophil granules [7,29,30,34,42,43]

Granule class	Peroxidase staining	Examples of contents	Mean density (g/mL)	Mean diameter (nm)	Appearance during neutrophil differentiation
Azurophil or primary	yes	• myeloperoxidase • defensins • BPI • leukocyte elastase • cathepsin G • proteinase 3 • azurocidin (CAP 37) • lysozyme • acid hydrolases • C5a-inactivating factor • serglycin proteoglycans	1.23	290	promyelocytic stage
Specific or secondary	no	• lactoferrin • collagenase • vitamin B₁₂ binding protein • lysozyme • NGAL • cathelicidins (e.g., CAP 18) • β_2-microglobulin • cytochrome b₅₅₈ • receptors (e.g., Mac-1, fMLP and CR-3)	1.19	210	myelocytic stage
Gelatinase or tertiary	no	• gelatinase	slightly < 1.19	111	metamyelocytic to band cell stage
Secretory vesicles or phosphasomes	no	• plasma proteins • alkaline phosphatase • superoxide anion	1.13	~75	band cell and mature neutrophil stage

glucose metabolism to supply NADPH for the "respiratory burst" (see section 1.4) [44].

1.4 - Neutrophil function

Neutrophils are critical for protecting us against the large variety of microorganisms to which we are routinely exposed. The importance of these cells in fighting infection can be exemplified by the rare human disorder known as Chronic Granulomatous Disease (CGD). Phagocytic cells (including neutrophils) from CGD patients have a defect in their "respiratory burst" (see below) and cannot generate adequate quantities of microbicidal radical oxygen species (ROS). As a result, these individuals suffer severe recurrent bacterial and fungal infections, especially in the lungs, lymph nodes, skin, liver and bones. In addition, vital organs may become blocked by granulomas formed from persistent low-level infections. Infections are most often caused by microorganisms such as gram-negative bacilli, *Staphylococcus aureus* (bacteria), *Candida*, *Aspergillus* or *Nocardia* (fungi). Infections can be fatal if not promptly treated [46,47]. In normal healthy individuals, circulating neutrophil counts average 4×10^6 per mL of blood [22]. During medical procedures that induce neutropenia (e.g., cancer chemotherapy), blood neutrophil counts must be monitored carefully. If the absolute neutrophil count falls below 5×10^5 per mL, then the risk of fatal infection becomes considerable [25]. Neutrophils accumulate rapidly at sites of infection, where they function as a first-line of defence. Without a proper neutrophil defence, we may succumb to a variety of microorganisms, even some that are not considered pathogenic [48].

The neutrophil has at its disposal a diverse arsenal of microbicidal functions to

combat invading microorganisms. These leukocytes collect at sites of infection by directed migration (chemotaxis). Neutrophils can migrate toward a chemoattractant at an average velocity of up to 20 $\mu\text{m}/\text{min}$ on flat surfaces [49]. Migration can occur even in a shallow gradient, consisting of a 1% rise in chemoattractant concentration across the length of the cell [50]. Molecules known to induce neutrophil chemotaxis include C5a [51], *N*-formylated hydrophobic peptides [52], leukotriene B4 [53], PAF [54], and the C-X-C chemokines IL-8/GCP-1, GCP-2, ENA-78, GRO α , GRO β and GRO γ [55-58]. The involvement of signal transduction pathways, actin polymerization and adhesion molecules in neutrophil chemotaxis has been well studied. However, the underlying mechanism responsible for cell movement is still not fully understood [59,60].

A circulating neutrophil first attaches lightly to capillary endothelium via the association of L-selectin (CD62L) on the neutrophil and L-selectin ligand on the endothelium [61,62]. In the presence of these interactions and normal blood flow, neutrophils "roll" along the endothelial surface. When rolling neutrophils encounter a chemotactic stimulus, stationary adhesion is maintained with local endothelial cells. This sustained adhesion occurs via ICAM-1 (CD54) at the endothelial cell-surface and by downregulating CD62L and upregulating CD11a/CD18 (LFA-1 β_2 integrin) and CD11b/CD18 (Mac-1 β_2 integrin) at the neutrophil cell-surface. Such modifications are mediated by proinflammatory agonists released at the site of infection [60,61]. The adherent neutrophil then enters the tissue by "squeezing" between adjacent endothelial cells (transendothelial migration), and then migrates to the site of infection by chemotaxis.

Once in the area of infection, the neutrophil can engulf or phagocytose

microorganisms that have been "opsonized". Opsonizing includes coating with immunoglobulin and/or complement factor 3b (C3b). Neutrophils bind opsonized particles via specific receptors (e.g., Fc γ RIIIb or Fc γ RI for immunoglobulin and CR1 or CR3 for C3b) [49]. Bound particles are engulfed by a movement around the particle that resembles locomotion, followed by a fusion of the plasma membrane at the leading edge. Once inside the cell, polymerized actin surrounding the phagosome is removed and the phagosome is fused with granules [63]. There are two main mechanisms of microbicidal activity available to the neutrophil at this point, namely oxygen-dependent and oxygen-independent mechanisms. Both mechanisms are thought to act synergistically.

Oxygen-dependent mechanisms involve the so-called "respiratory burst". A multiprotein complex termed the phagocyte oxidase or NADPH oxidase is assembled at the phagolysosomal membrane surface. Using this oxidase, electrons are shuttled from intracellular NADPH (supplied via the hexose monophosphate shunt) to molecular oxygen to form superoxide anion in the phagolysosomal lumen. Superoxide can dismutate either spontaneously or via enzyme catalysis (superoxide dismutase) to form hydrogen peroxide (H₂O₂). In the presence of Cl⁻, myeloperoxidase converts H₂O₂ into hypochlorous acid (i.e., bleach), which can react with amines to form chloramines. Any of these compounds can be toxic to a microorganism, although the latter two are the most potent [47,64]. The neutrophil also possesses oxygen-independent microbicidal activities including hydrolytic enzymes that degrade bacterial macromolecules and antimicrobial cationic proteins. One the best characterized of the latter is the family of peptides known as defensins [37]. Defensins are 29-35 amino acid, cysteine- and arginine-rich peptides, representing >5% of the total cellular

protein in human neutrophils. Purified defensins exhibit toxicity *in vitro* against many organisms, including gram-positive and gram-negative bacteria, fungi, and enveloped viruses. Membrane permeabilization, thought to be caused by the formation of voltage-regulated channels in lipid bilayers, is the lethal event [37,65-67].

1.5 - Apoptosis in mature neutrophils

Peripheral blood neutrophils undergo apoptosis spontaneously when cultured *in vitro*. Literature accounts vary, but most report that 50% of the cells become apoptotic after 10 to 40 h in culture [68-74]. Apoptotic neutrophils have also been detected *in vivo* in inflamed tissue of the joint, kidney, lung and gut [68,75-77]. Aged neutrophils exhibit typical characteristics of apoptosis including DNA fragmentation into nucleosome-sized pieces, intense chromatin condensation, rounding of the nucleus, cell shrinkage, intracellular acidification (lowering of pH from 7.2 to 6.2), formation of cytoplasmic vacuoles and flipping of phosphatidylserine to the outer leaflet of the plasma membrane [68,74,78-81]. Changes related to the specialized functions of these cells also occur. Neutrophils undergoing apoptosis show a marked loss of microbicidal functions. They can no longer effect shape changes, chemotaxis, degranulation and respiratory burst activity in response to a receptor-mediated stimulus (e.g., fMLP), although the respiratory burst can still be invoked by direct activation of PKC (i.e., with PMA) [82,83]. Apoptotic neutrophils lose the capacity to phagocytose opsonized zymosan [82] and adhesion to E-selectin and fibronectin is reduced [84]. β/γ -actin is cleaved into 30 and 25 kDa fragments [85] and membrane-associated actin (which links polymerized actin to the plasma membrane) is

cleaved between Val⁴³ and Met⁴⁴ [86]. Activation of caspase-3 has been shown to occur in aged neutrophils [87], but a calpain-like protease was implicated in cleavage of membrane-associated actin [86]. Also, low-affinity IgG receptors (called CD16 or FcγRIIIb), fMLP receptors and L-selectin are lost from the cell surface, possibly by metalloprotease activity [73,80,82,84,88]. However, not all cell-surface molecules are lost since CD11b/CD18 and CD11c/CD18 β₂ integrins have been found at increased levels in apoptotic neutrophils [84]. In general, it is believed that the changes seen in aged neutrophils serve to render them functionally inactive so that, after killing invading microorganisms, they can be cleared from a tissue or inflammatory environment without damaging host cells.

Although neutrophil apoptosis does occur spontaneously, numerous agents have been shown to accelerate or delay the process. Known accelerators include TNF-α [89-91], fMLP [89], Fas ligand [92-94], phagocytosis of opsonized particles [95], phagocytosis of *Escherichia coli* [96], extracellular proteases such as neutrophil elastase [97], engagement of β₂ integrins (CD11/CD18) [98], erythromycin [99], sodium arsenite [100], ROS [89,96,101] and the translation and transcription inhibitors, cycloheximide and actinomycin D [102,103]. Agents that can delay the onset of apoptosis include IL-2 [78], IL-4 [85], IL-8 [104], IL-15 [105], GM-CSF [70,71,74], G-CSF [79], LPS, C5a [74,91], glucocorticoids [106-108], nicotine [109], ATP, diadenosine triphosphate, diadenosine tetraphosphate [110], agents that elevate cytosolic Ca²⁺ levels [72], and agents that elevate cAMP levels [111]. Some of these agents (e.g., nicotine and erythromycin) are of specialized or nonphysiologic significance, but many others are important mediators of neutrophil survival within the inflammatory environment. The presence of multiple proinflammatory mediators at the site

of an infection can influence neutrophil survival. For example, LPS can delay the onset of apoptosis, but the presence of IL-10 can inhibit that delay [112]. LPS or PAF can also inhibit apoptosis induced by TNF- α [91,113]. Another example is the effect of IL-6, which has been reported to both accelerate and delay neutrophil apoptosis. This discrepancy was later explained by Biffl *et al.*. They found that the delay of apoptosis observed with IL-6 occurred only at high PMN concentrations ($10\text{--}20 \times 10^6$ cells/mL), possibly involving autocrine PAF activity [114-116]. In general, the net effect of proinflammatory mediators is to preserve neutrophil microbicidal activity by delaying apoptosis in the early stages of acute inflammation (<48 h). This extends the time that neutrophils are available to fight the infection.

Programmed cell death is delayed but not prevented in mature neutrophils by proinflammatory mediators. Once an infection has been managed, apoptosis proceeds and resolution of the inflammation begins (>48 h). The average half-life of a neutrophil can be extended by 6-32 h when cultured *in vitro* with ≥ 0.1 ng/mL GM-CSF [69,71,74], although one group reported a survival of >9 d with the same cytokine [70]. **GM-CSF-induced delay can be abrogated by cotreatment with cycloheximide or actinomycin D, indicating its dependence on protein and RNA synthesis.** Other agents that require transcription/translation to delay neutrophil apoptosis include dexamethasone [107], LPS [91], IL-4 [85] and G-CSF [79,117]. The importance of transcription/translation can also be illustrated using sodium butyrate. Butyrate, a pharmacologic agent that increases gene expression possibly by inhibiting histone deacetylase and decompacting chromatin, also delays neutrophil apoptosis [118]. The protein(s) responsible have not yet been identified,

although upregulation of the 57-kDa cytosolic B subunit of the v-ATPase was recently implicated in G-CSF-induced delay [79,117]. Mature neutrophils express pro-apoptotic Bax protein, but express undetectable levels of anti-apoptotic Bcl-2, Bcl-X_L and Mcl-1 [20]. Apparently Bcl-2 family members are not involved in cytokine-induced apoptotic delay; GM-CSF was recently shown to not effect Bcl-2 levels [16], although other family members were not tested. Further study is needed to identify the protein(s) responsible for receptor-mediated apoptotic delay in these cells. Whether the pathways involved are specific to the neutrophil or are more general, the information gained will further our understanding of both neutrophil physiology and apoptotic processes.

There exists increasing evidence to implicate R $\ddot{O}$ S in the progression of programmed cell death in neutrophils. Spontaneous and fMLP-induced apoptosis can be inhibited by exogenously added superoxide dismutase [89]. The increased apoptosis observed in neutrophils after phagocytosis of *E. coli* can be abrogated by agents that increase intracellular glutathione levels [96]. Neutrophils from CGD patients exhibit slower rates of spontaneous apoptosis than normal [101], and hypoxia has also been shown to reduce the rate of spontaneous apoptosis in neutrophils from normal donors (lowering it at 20 h from $78.7 \pm 2.2\%$ in air to $23.1 \pm 3.2\%$ in anoxic conditions) [119]. Thiol oxidizing and alkylating agents such as diamine and diethylmaleate were shown to accelerate neutrophil apoptosis *in vitro*, an effect that was reversed by adding N-acetylcysteine, genistein or herbimycin A [120]. This suggested that intracellular redox-sensitive mechanisms, possibly involving glutathione and tyrosine phosphorylation, were involved in induction of apoptosis in these cells. A redox-sensitive signal transduction pathway has also been implicated in Fas-mediated

neutrophil apoptosis [92,101]. However, not all receptor-mediated effectors exert their influence via ROS, since apoptosis induced by TNF- α could not be blocked by exogenously added superoxide dismutase [89]. Apparently, ROS are important not only for neutrophil bactericidal activity but also for regulating the rate of apoptosis within the inflammatory environment. Although not investigated in this thesis, the effect of cytokines on the level of redox-protective enzymes would be an interesting area of study.

1.6 - Clearance of apoptotic neutrophils from tissues

Senescent neutrophils must be removed from a tissue before they disintegrate and release noxious substances into the extracellular milieu. In the late 19th century, Metchnikoff had observed macrophages ingesting intact neutrophils at the site of experimentally-induced inflammation [26]. In 1982, Newman *et al.* found that human monocyte-derived macrophages and rabbit inflammatory macrophages could phagocytose only human neutrophils that were aged in culture; freshly isolated neutrophils were refractory to macrophage engulfment [121]. The mechanism by which macrophages remove apoptotic neutrophils *in vivo* has not been addressed to date. However, using *in vitro* experiments, the strongest candidates for macrophage recognition of intact senescent neutrophils are the putative phosphatidylserine receptor and the vitronectin receptor. Receptor usage seems to depend on the type of macrophage [122]. Neutrophils (and other cells) translocate anionic phosphatidylserine to the outer leaflet of the plasma membrane during the early stages of apoptosis [81,123,124]. The phosphatidylserine receptor on the surface of macrophages remains unknown, but candidates include types A and B scavenger receptors [125,126] and

the oxidized low density lipoprotein receptor (CD68) [127].

Removal of senescent neutrophils via the vitronectin receptor in human monocyte-derived macrophages is the most well understood, first being reported by Savill *et al.* in 1989 [128]. The proposed model for binding involves the RGD-dependent $\alpha_v\beta_3$ vitronectin receptor integrin and CD36 (amino acids 155-183) on the macrophage cell-surface, and unknown anionic structures on the neutrophil cell-surface. In addition, the secreted glycoprotein thrombospondin 1 (TSP1) is necessary to act as a bridging molecule between the macrophage and neutrophil binding sites [129-131]. Once the neutrophil binds the macrophage, phagocytosis occurs rapidly, followed by intracellular degradation of the apoptotic neutrophil. This method of phagocytosis prevents the release of proinflammatory agonists from the macrophage, allowing cessation and resolution of the inflammatory response [132-134]. In addition to macrophages, fibroblasts [135] and glomerular mesangial cells [136] have also been shown to phagocytose apoptotic neutrophils by similar mechanisms.

1.7 - GM-CSF and its receptor

Granulocyte-macrophage colony-stimulating factor (GM-CSF, also called CSF-2) is a small, heterogeneously glycosylated protein of 127 amino acids and an apparent molecular mass of 18-24 kDa on SDS-PAGE [137]. GM-CSF was cloned based on its ability to induce the formation of granulocyte-macrophage, granulocyte and macrophage colonies from bone marrow cultured in semi-solid media [137-139]. Human GM-CSF, although species-specific, is 60% homologous to murine GM-CSF at the amino acid level

[137]. Despite the proliferating and differentiating effect of the cytokine on hematopoietic precursor cells, mice homozygous for a disrupted GM-CSF gene (i.e., GM-CSF knockout mice) show no major abnormalities of hematopoiesis [140,141]. This may be due to compensatory mechanisms whereby other cytokines such as Steel factor (*c-kit* ligand) and G-CSF can substitute for the missing GM-CSF [140,142]. In support of this view, a recently developed GM-CSF/G-CSF double knockout mouse demonstrated greater phenotypic abnormalities than those of either single knockout [141].

Mature human neutrophils, eosinophils, monocytes and endothelial cells possess GM-CSF receptors and respond to the cytokine [143,144]. By Scatchard analysis, mature neutrophils have an average of 540 GM-CSF binding sites per cell, with a high affinity K_d of 20-100 pM or 0.4-2 ng/mL [143,145]. Promyelocytic and myelocytic neutrophil precursors will proliferate in response to GM-CSF, but they have fewer receptors than their mature neutrophilic counterparts. Differentiation of promyelocytic HL-60 or AML cells to granulocytic cells with either DMSO or all *trans*-RA respectively causes an increase in GM-CSF receptor number or affinity [145,146]. Also despite the lack of abnormal hematopoiesis in GM-CSF knockout mice, these animals do have an increased propensity to develop both lung and soft-tissue infections [140,141]. Taking all these data into consideration, it appears that GM-CSF is more important in the functioning of mature neutrophils than in their development within the bone marrow.

The responses of mature neutrophils to GM-CSF are numerous and are summarized in Table 1.II. Responses can be categorized either as direct effects that require only GM-CSF stimulation or as indirect effects that require an additional stimulus. An indirect effect

is usually an augmented response to a secondary stimulus (e.g., fMLP) that occurs after the cell has been "primed" with GM-CSF. GM-CSF can be secreted by hematopoietic (monocytes-macrophages and lymphocytes) and nonhematopoietic (fibroblasts, endothelial cells and some epithelial cells) cell-types in response to various proinflammatory mediators such as LPS, IFN γ , IL-1 α , IL-1 β and TNF [147]. GM-CSF mRNA is highly unstable due to the presence of AU-rich sequence elements in its 3'-UTR. Proinflammatory mediators often regulate GM-CSF mRNA levels post-transcriptionally by enhancing mRNA stability [147-149]. Thus, cells residing at the site of an infection can be induced to secrete GM-CSF, which will modulate neutrophil functions according to the criteria of Table 1.II. In general, GM-CSF enhances microbicidal activity and prolongs the neutrophil lifespan to fight the infection. However, the presence of other locally-produced cytokines can complicate matters by agonizing or antagonizing the effect of GM-CSF. Although one should remain aware of these synergistic effects, they are not a consideration of this thesis.

Table 1.II
Effects of GM-CSF on mature neutrophils [145,150-154].

DIRECT EFFECTS

- prolonged survival (delay of apoptosis)
- membrane ruffling
- random migration
- increased adhesion to surfaces
- increased CD11b/CD18, CD11c/CD18, fMLP receptors, FcγRI, CR1 and CR3 on the cell-surface
- release of arachidonic acid and leukotriene B4
- intracellular alkalinization
- increased RNA and protein synthesis
e.g., FLAP, *c-fos*, IL-3 receptor α , 5-lipoxygenase, IL-8, IL-1Ra [15,155-159]

INDIRECT EFFECTS (PRIMING)

- increased chemotaxis
- enhanced oxidative metabolism
- increased degranulation
- increased phagocytosis and antimicrobial activity
- increased release of arachidonic acid and leukotriene B4
- enhanced antibody-dependent cytotoxicity

The high-affinity GM-CSF receptor is a heterodimeric transmembrane protein consisting of a cytokine-specific α chain and a β_c chain that is common to receptors for GM-CSF, IL-3 and IL-5 [142,160]. The β_c chain is mostly responsible for signal transduction in response to ligand binding. Engagement of GM-CSF results in intracellular tyrosine phosphorylation of the receptor and other proteins [161], although the receptor itself lacks intrinsic kinase activity [147,162]. In hematopoietic cells, the β_c chain cytoplasmic region possesses domains that seem to signal for both cell survival (anti-apoptosis) and

proliferation, although mitogenic signals obviously do not function in mature neutrophils [142]. GM-CSF is believed to upregulate *c-fos* in neutrophils by activation of JAK2 and subsequent activation of STAT5B [163]. Lyn, a member of the Src family of tyrosine kinases, is known to associate with the GM-CSF receptor. Lyn has been implicated (using antisense experiments) in GM-CSF-induced apoptotic delay [16], possibly acting as an upstream activator of Ras [142]. GM-CSF also increases the intracellular concentration of cGMP, a second messenger implicated in augmenting neutrophil responses to fMLP (i.e., priming) [164-166]. Two additional kinases known to be activated in GM-CSF-stimulated neutrophils are phosphatidylinositol 3-kinase [167] and myosin light chain kinase [154]; the latter has been implicated in GM-CSF-induced random migration.

1.8 - The significance of transcription and translation in neutrophils

For many years it was believed that synthesis of RNA and protein in neutrophils was of little significance to their normal functioning. Neutrophils still have the capacity to phagocytose particles when treated with inhibitors of transcription and/or translation [168]. Microbicidal compounds are synthesized earlier in differentiation and stored in granules [1,169]. Also, electron micrographs of neutrophils reveal few ribosomes, scarce ER and Golgi, no visible nucleoli and highly heterochromatic DNA [1,25,38]. Granelli-Piperno *et al.* were the first to realise the importance of transcription and translation in certain neutrophil functions [170]. They found that the secretion of plasminogen activator by human PMNs was substantially reduced by RNA and protein synthesis inhibitors, and they later verified by radiolabeling experiments that these cells do indeed synthesize small, but

detectable quantities of RNA and protein [171]. The majority of subsequent reports studying neutrophilic mRNA and protein species were related to the specialized functions of these cells (e.g., proinflammatory agonists, phagocyte receptors, etc.). The amount of proinflammatory mediators secreted per cell is often low compared to those of other leukocytes such as monocytes/macrophages. For this reason, mediators secreted by neutrophils are often dismissed as functionally insignificant. However, due to the accumulation of large numbers of neutrophils in acute inflammation, the local concentration of secreted proinflammatory mediators can become significant [172].

I became aware initially of the importance of transcription/translation in neutrophils when Brach *et al.* demonstrated that actinomycin D or cycloheximide could abrogate the apoptotic delay induced by GM-CSF [70]. GM-CSF is arguably the most potent proinflammatory mediator and stimulates multiple neutrophil functions (see Table 1.II). Not all of these effects require RNA and protein synthesis, but the increase in cell survival apparently does. We believed that the low "background" of transcription and translation in neutrophils would simplify the identification of novel GM-CSF-responsive genes. Neutrophils, albeit terminally differentiated and highly specialized cells, must carry out at least some functions of "normal" cells. The expectation was to identify genes involved both in specialized neutrophil functions and in more general cell functions. Arbitrary cloning techniques such as DDRT-PCR (used in chapter 4) would allow the identification of both types of transcripts.

1.9 - Objective of thesis and general outline

The objective of this thesis work is to identify novel aspects of transcription and translation in *in vitro* cultured human peripheral blood neutrophils with an emphasis on the effect of GM-CSF-stimulation. The results are divided into four chapters.

- Chapter 3 -- To verify that the isolated neutrophils are responding appropriately to GM-CSF, a number of known effects of the cytokine from Table 1.II were tested.
- Chapter 4 -- RNA synthesis in response to GM-CSF was studied. Firstly, the effect of the cytokine on general RNA metabolism using [³H]uridine radiolabeling was analysed. DDRT-PCR was then used to identify differentially expressed transcripts. Two of these transcripts (*hsgk* mRNA, upregulated by GM-CSF, and *hsgly* intron 2, not affected by cytokine stimulation) were analysed in more detail.
- Chapter 5 -- Protein synthesis in response to GM-CSF was studied. Using immunoblotting, the effect of the cytokine on expression of pro-apoptotic and anti-apoptotic Bcl-2 family members was addressed. The effect of GM-CSF on general protein metabolism using [³⁵S]methionine radiolabeling was then analysed. By electrophoresis of radiolabeled proteins on SDS-PAGE, an unknown 26-27 kDa protein (named p27gran) was identified as demonstrating the largest increase in translation with GM-CSF-stimulation. An unsuccessful attempt was then made to identify this protein.
- Chapter 6 -- An mRNA encoding a protein of unknown function (i.e., hTegt, identified in chapter 4) that is unaffected by GM-CSF stimulation but highly expressed in

human granulocytes was studied further at the protein level. Toward this end other cultured human cells were used.

Chapter 2

General Materials and Methods

2.1 - Purification of human granulocytes

Granulocytes were isolated from the peripheral blood of normal, consenting donors similarly to that described previously [173]. Blood was collected by venous puncture into 10 mL Vacutainers (tripotassium EDTA as anticoagulant) and was diluted by adding ½ volume of 137 mM NaCl, 5 mM KCl, 0.8 mM MgSO₄, 5 mM Glc and 10 mM Hepes/NaOH (pH 7.4). The diluted blood was centrifuged through a cushion of Ficoll-Diatrizoate (Lympholyte-H) at $720 \times g$ for 15 min at room temperature. The resulting upper layer (plasma and platelets) was removed, clotted by addition of CaCl₂ to 5 mM, filter sterilized and used as autologous serum. The mononuclear band and the Ficoll-Diatrizoate layers were aspirated and discarded, leaving the bottom pellet of erythrocytes and granulocytes. Contaminating erythrocytes were removed by two sequential lysis steps. (i) NH₄Cl treatment at room temperature, and (ii) ⅓×isotonicity for 60 s on ice. (Isotonicity was restored after the 60 s incubation by adding 2 mL of 1.7 M NaCl per 30 mL of cell suspension.) After each lysis step, the samples were centrifuged at 4°C for 10 min at $800 \times g$, and the supernatant discarded. Purified granulocytes were suspended in 5 mL appropriate media, and cell counts determined using a Coulter Counter (Coulter Electronics of Canada Ltd., Burlington, ON). All granulocyte incubations were performed at a concentration of 10^6 cells/mL of RPMI 1640 + 10% autologous serum at 37°C in a humidified, 5% CO₂ atmosphere unless otherwise noted. With the exception of those in Appendix I, all granulocyte isolations and incubations were performed under aseptic conditions. Typical granulocyte preparations consisted of 96% neutrophils, 3% eosinophils, <1% lymphocytes and <1% monocytes (with variable erythrocyte contamination) as determined by microscopic examination. Although

basophilic granulocytes comprise ~1% of circulating PMNs, they were not isolated with this procedure because their average buoyant density is less than that of Ficoll-Diatrizoate (i.e., less than $1.077 \text{ g}\cdot\text{cm}^{-3}$) [1,174].

2.2 - Purification of human mononuclear cells

If required, mononuclear cells (lymphocytes and monocytes) were isolated during the granulocyte purification outlined in section 2.1. The mononuclear band, positioned at the interface between the plasma and Ficoll-Diatrizoate layers, was removed and was diluted to 40 mL with ice-cold PBS. The suspension was centrifuged at $250 \times g$ for 10 min at 4°C . The supernatant (containing platelets) was discarded, and the pellet (enriched in mononuclear cells) was suspended in another 40 mL ice-cold PBS. The suspension was centrifuged again at $250 \times g$ for 10 min at 4°C . The supernatant was discarded and the pellet suspended in 5 mL buffered saline or media. Cell counts were determined with a hemacytometer.

2.3 - Maintenance of cell lines

Cell lines were maintained in DMEM + 10% FCS at 37°C in a humidified, 5% CO_2 atmosphere. HL-60 human promyelocytic leukemia cells, HeLa human epitheloid cells, and 293T human kidney epithelial cells were utilized. HeLa and 293T were maintained in 10 mL media in 10-cm tissue culture plates; prolonged postconfluency was avoided. These adherent cells were split ~1:10 every 3 d by treating with trypsin/EDTA and diluting into fresh media. HL-60 cells were maintained in 20-30 mL media in 15-cm tissue culture plates at $\leq 0.5\text{-}1 \times 10^6$ cells/mL. Cells were collected every 3 d, pelleted by centrifugation at 2000 rpm (room

temperature, 5 min), and diluted ~1:10 in fresh media. All cell counts were performed with a hemacytometer.

2.4 - Isolation of total RNA

2.4.1 - Guanidinium-acid phenol method

To isolate full-length RNA from granulocytes, a modification of the method of Chomczynski and Sacchi was used [175]. Granulocytes were collected, then pelleted by centrifuging at 2000 rpm for 5 min (4°C). One mL 4 M guanidinium thiocyanate, 0.5% N-lauroylsarcosine, 0.1 M 2-mercaptoethanol and 25 mM sodium citrate/HCl (pH 7) per 10⁶ cells was added to the cell-pellet, and triturated well with a plastic transfer pipette. The following components (per 10⁶ cells) were added sequentially, mixing by inversion after each addition. (i) 100 µL 2 M sodium acetate/acetic acid (pH 4), (ii) 1 mL water-saturated phenol, and (iii) 200 µL chloroform:isoamyl alcohol (49:1). The mixture was chilled on ice (15 min) then centrifuged for 20 min at 9000 rpm and 4°C in a swing-out bucket rotor (Sorvall HB-6). The upper phase was collected, mixed with 50 µg glycogen and precipitated with 2 volumes absolute ethanol for 2 h at -20°C. The faint precipitate was pelleted by centrifuging as before, and washed with absolute ethanol. The pellet was allowed to air-dry, dissolved in 300 µL 1 M urea, 1% SDS, 0.5 M LiCl, 5 mM CDTA, 100 µg/mL proteinase K and 40 mM Mops/NaOH (pH 6.8) (RES) and digested at 37°C for 30 min. The mixture was extracted once with phenol/CHCl₃ (1 g phenol per mL CHCl₃), and back-extracted with 100 µL RES without protease. The pooled aqueous phase was extracted once with CHCl₃. To the aqueous phase was added an equal volume of 3 M LiCl/40% (v/v) ethanol, and the mixture

allowed to stand overnight in ice-water. The sample was centrifuged at room temperature for 4 min at $13,000 \times g$. The supernatant was carefully discarded, and the faint precipitate dissolved in 50 μ L 0.1% SDS, 1 mM CDTA, 10 mM sodium phosphate (pH 6.8). The RNA was precipitated for at least 20 min (-20°C) with 2 volumes absolute ethanol and 0.1 volume sodium acetate (pH 5.6). The RNA was pelleted by a microcentrifuge spin, washed with 70% ethanol, dried, and dissolved in an appropriate volume of 1 mM CDTA, 10 mM sodium phosphate (pH 6.8).

2.4.2 - SDS-urea method

To isolate RNA from cell-lines or if the integrity of granulocyte RNA was less of a concern (e.g., assessing radioactive incorporation only), then a more rapid method was employed as described previously [176]. If quantification of RNA was required, its absorbance at 260 nm was measured.

2.5 - Northern blotting

RNA (typically about 2-6 μ g per lane) was electrophoresed on a 1-1.2% agarose gel with formaldehyde as described previously [177], then was transferred to nylon membrane (Hybond-N) in $10\times\text{SSC}$ (1.5 M NaCl, 0.15 M sodium citrate/HCl (pH 6)) by either vacuum or capillary transfer. Membranes were washed in $10\times\text{SSC}$, air-dried, then fixed by baking for 2 h at 80°C in a desiccated oven. Membranes were prehybridized for 1-4 h at 42°C in bottles with 50% formamide, $6\times\text{SSC}$, 0.5% SDS, 50 μ g/mL denatured salmon DNA, $5 \times$ Denhardt's solution (0.1% BSA, 0.1% Ficoll, 0.1% polyvinylpyrrolidone) and 50 mM sodium phosphate (pH 6.8). Radioactive probe was prepared by random priming using [α -

^{32}P]dCTP and a random priming kit, following manufacturer's instructions. Heat-denatured probe was added to the bottle and allowed to hybridize at 42°C overnight. Membranes were washed twice at low stringency (2×SSC, 0.1% SDS at 42°C, 20 min each), and twice at high stringency (0.16×SSC, 0.1% SDS, 10 mM sodium citrate (pH 6.8) at 65°C, 20 min each). Damp membranes were exposed to Kodak X-OMAT AR x-ray film or to PhosphorImager screen (PhosphorImager SI or Storm 860, Molecular Dynamics, CA) to visualize. If a membrane was to be reprobed, it was stripped of bound probe by shaking in boiling 0.1% SDS until the solution cooled to room temperature.

2.6 - Probing Northern blots with human 28S rRNA

To normalize loading on granulocyte Northern blots, stripped blots were probed with a random primed cDNA fragment representing nucleotides 1405-4933 of human 28S rRNA (GenBank accession #M11167). Prehybridization, hybridization, washing and visualizing procedures were followed from section 2.5. Because the 28S signal was very intense and could not be removed entirely, this procedure was performed after all other probings were completed.

2.7 - Visualizing 28S and 18S rRNA on nylon membranes

All steps were carried out at room temperature. Stripped blots were washed for a few min in absolute ethanol to remove residual SDS. Blots were stained for 5 min in 0.1% methylene blue in 50% ethanol, and were destained in water until rRNA bands could be clearly seen. Stained blots were allowed to air-dry, and the positions of the 28S and 18S

bands noted.

2.8 - Western blotting

Protein extracts (5-25 μ g total protein per lane) were electrophoresed on 12% discontinuous SDS-polyacrylamide gels by the method of Laemmli [178]. Resolving gels were removed from the glass plates and equilibrated in 10 mM NaHCO₃, 3 mM Na₂CO₃, 10% methanol (transfer buffer) for 20 min. Proteins were transferred electrophoretically to PVDF membranes (Immobilon-P) in a Bio-Rad Trans-Blot Cell with cooled transfer buffer overnight at 14 V. Membranes were stained with 0.5% (w/v) Ponceau S in 1 % (v/v) acetic acid, then destained in water to verify integrity of loaded protein and/or visualize protein molecular weight markers. Membranes were then treated sequentially with the following at room temperature with gentle mixing. (i) 2 $\times$ 5 min washes with TBST (0.15 M NaCl, 0.1% (v/v) Tween 20 and 10 mM Tris/HCl (pH 8.0)), (ii) 1% BSA for 30 min, (iii) primary antibody appropriately diluted in TBST for 1-2 h, (iv) 4 $\times$ 5 min washes with TBST, (v) alkaline phosphatase conjugated secondary antibody appropriately diluted in TBST for 1 h, and (vi) 4 $\times$ 5 min washes with TBST. Immunoreactive bands were visualized by developing the membranes in 0.1 M NaCl, 5 mM MgCl₂, 0.1 M Tris/HCl (pH 9.5), 25 mg/mL BCIP and 50 mg/mL NBT at room temperature. To stop the reaction, membranes were washed once in 0.5% acetic acid and once in water.

2.9 - Quantifying total protein

Total protein was quantified in cellular extracts using the amine-reactive fluorophore

fluorescamine [179]. An aliquot of cellular extract (typically 1-2 μ L) or a known mass of BSA (typically 0-20 μ g) was diluted into 1.5 mL 3 mM CDTA, 0.1 % SDS, 0.2 M sodium borate (pH 9.2) in a 13 $\times$ 100 mm disposable glass culture tube. One mL of fluorescamine solution (0.15 mg/mL acetone) was added while vortexing, and the solution allowed to stand at room temperature for $\geq$ 3 min. 0.5 mL water was then added, and the fluorescence read on a Perkin-Elmer LS-5 Fluorescence Spectrophotometer (excitation wavelength = 390 nm, emission wavelength = 475 nm). Protein concentration in extracts was calculated using a standard curve that had been constructed from the BSA values. Since fluorescamine reacts with primary amines, buffers containing Tris were avoided in this assay.

2.10 - *In vitro* transcription and translation

I.M.A.G.E. clone #207657 (hTEGT) was linearized after the stop codon by *Hind*III digestion. Capped hTEGT mRNA was synthesized from 0.5 μ g linearized plasmid using the T7 Cap-Scribe *in vitro* transcription kit according to the manufacturer's instructions. One tenth of the *in vitro* transcribed mRNA was then subjected to *in vitro* translation using either rabbit reticulocyte or wheat germ lysates according to supplied instructions. L-[35 S]methionine was used as the radiolabeling amino acid. If needed, 3 μ L of canine pancreatic microsomes were added per *in vitro* translation reaction. After completion, the reaction was digested with 200 μ g/mL pancreatic RNase for 5 min at 25-30°C to stop the reaction and eliminate radiolabeled tRNA.

2.11 - Isolation of genomic DNA

HL-60 cells or human granulocytes were collected and were washed once with PBS. Cells were suspended in a small volume of PBS, and were lysed by addition of 1 M LiCl, 1 M urea, 0.2% SDS, 5 mM CDTA and 50 mM Tris/HCl (pH 8.0) (DES, 0.3 mL per 10^6 cells) with gentle vortexing. The extract was digested with 100 μ g/mL proteinase K at 45°C for at least 1 h. The subsequent procedure varied depending on the cell-type used.

2.11.1 - HL-60 cells

DNA was precipitated with 0.55 volumes isopropanol at room temperature. When DNA fibres appeared, the precipitate was collected by low-speed centrifugation (1000-2000 $\times$ g, 10 min, room temperature) and was dissolved in 1 mM CDTA, 10 mM Tris/HCl (pH 7.5) (CT, 50-100 μ L per 10^6 cell equivalents). The solution was digested with 50 μ g/mL DNase-free pancreatic RNase for 10 min at 37°C, and was then diluted with an equal volume of DES. The mixture was extracted twice with an equal volume of phenol/ CHCl_3 (1 g phenol per mL CHCl_3) and once with an equal volume of CHCl_3 , with gentle shaking. DNA was precipitated at room temperature with 1.5 volumes ethanol. The precipitate was collected by centrifugation, washed with 70% ethanol, and briefly air-dried. The DNA was dissolved in CT, and its concentration calculated by absorbance at 260 nm.

2.11.2 - Human granulocytes

The solution was extracted twice with 300 μ L phenol/ CHCl_3 (1 g phenol per mL chloroform), and once with 300 μ L CHCl_3 with gentle shaking. DNA was precipitated from the aqueous phase with 2 volumes ethanol at -20°C for at least 30 min, and was collected by centrifugation at 10,000 rpm for 10 min at 4°C. The pellet was washed with 70% ethanol,

was allowed to air-dry briefly, and was dissolved in 200 μ L CT. The concentration of DNA was estimated by assuming DNA content at 6 μ g per 10^6 cells.

2.12 - Isolation of plasmid DNA

2.12.1 - Mini-prep

Plasmid was isolated from 1 mL of overnight *E. coli* culture by the alkaline lysis method of Birnboim and Doly [180]. The final ethanol-precipitate was dried under vacuum and was dissolved in 50 μ L water. Contaminating RNA was removed by digestion with 100 μ g/mL DNase-free pancreatic RNase for 30 min at 37°C before analysis of the plasmid by restriction analysis and agarose gel electrophoresis.

2.12.2 - Maxi-prep

Plasmid was isolated from 250 mL of overnight *E. coli* culture by alkaline lysis and binding to glass fibre, as previously described by Birnboim [181]. DNA concentration was determined by measuring its absorbance at 260 nm in a spectrophotometer.

2.13 - DNA sequencing

DNA fragments were sequenced either manually using the Sequenase Version 2.0 DNA Sequencing Kit according to supplied instructions or were submitted to a Sequencing Facility (Cancer Research Group, Ottawa Regional Cancer Centre) for automated sequencing.

2.14 - Purification of DNA fragments by agarose gel electrophoresis

DNA mixtures were electrophoresed on 0.8% agarose until the bromophenol tracking dye had migrated $\frac{2}{3}$ - $\frac{3}{4}$ the length of the gel (electrophoresis buffer was 1 mM CDTA, 20 mM sodium acetate, 40 mM Tris/acetic acid (pH 7.8)). The gel was stained at room temperature for 20 min with 0.1 μ g/mL ethidium bromide in water, visualized under UV transillumination, and the band(s) of interest excised. Excised bands were dissolved in 9 M sodium perchlorate, 15 mM CDTA, 75 mM Tris/HCl (pH 8.0) at room temperature (2 mL/g agarose). DNA fragments were then purified by binding to glass powder, using reagents similar to those in the QIAquick Gel Extraction Kit. Eluted DNA was quantified by fluorescence on an SSF-600 Solid State Fluorimeter (Tyler Research Corp., Edmonton, Alberta), using PicoGreen reagent according to manufacturer's instructions. Sonicated human leukocyte DNA that had been accurately quantified by UV absorption was used as a standard.

2.15 - Bacterial transformation

2.15.1 - Heat-shock method

E. coli (DH5 α , XL-1 Blue, or Sure) were rendered competent by standard methods and frozen [182]. Ligation mixture or purified plasmid (<20 μ L) was mixed gently with 125 μ L thawed competent bacteria in a plastic culture tube. The tube was allowed to stand on ice for 20 min, and was then transferred to a 42°C water bath for 90 s. LB broth, 4 mM MgSO₄, 10 mM KCl was added (500 μ L) and the tube shaken in an incubator at 250 rpm for 90 min at 37°C.

2.15.2 - Electroporation

Log-phase *E. coli* (DH5 α , XL-1 Blue, or Sure) were prepared by washing the cells twice with 10% glycerol, resuspending them at $\sim 10^{11}$ cells/mL in 10% glycerol, freezing them in aliquots in liquid N₂, and storing them at -80°C. Prior to use, ligation mixtures or plasmids were precipitated by ethanol and dissolved in water to remove salts that could interfere with electroporation. Thawed competent bacteria (20 μ L) were mixed with 1 μ L DNA in a 0.5 mL microcentrifuge tube. Using a micropipetter, the bacterial/DNA suspension was placed between the bosses of a sterile disposable Micro-Electroporation chamber. The cells were electroporated once in a Cell-Porator with Voltage Booster (Life Technologies Inc., Burlington, Ontario) using the following settings:

Low Ω

Fast Charge Rate

Capacitance = 300 μ F

Voltage Booster Resistance = 4 K Ω

Voltage = 408 V.

The electroporation chamber containing the bacterial suspension was kept on ice at all times. Bacteria were removed from the chamber by rinsing with 1 mL 2 $\times$ YT media, transferred to a plastic culture tube, and incubated for 1 h at 37°C with shaking. Typically 20 and 100 μ L aliquots were then spread on LB agar containing the appropriate antibiotic, and plates were incubated inverted at 37°C overnight.

2.16 - PCR

Unless otherwise noted, PCR reactions contained 20 mM Tris/HCl (pH 8.5), 50 mM KCl, 0.05% Tween 20, 2.5 mM MgCl₂, 0.3 mM of each dNTP, 50 pmol forward primer, 50 pmol reverse primer, DNA template, and 1 unit *Taq* DNA polymerase in a final volume of 50 μ L. All reagents were added to a 0.5 mL plastic microcentrifuge tube (except *Taq*) and the liquid overlaid with 2-3 drops of silicone oil (DC 200/200, density = 0.97 g/mL at 20°C). Tubes were placed in the PCR machine (MJ Research Inc. Programmable Thermal Controller, Hypercell Biologicals, AECL, Chalk River, Ontario), were heated to 95°C for 5 min, and were held at 72°C while *Taq* DNA polymerase was added underneath the silicone oil. Specific cycling conditions are given with each fragment amplified in this thesis. At the end of the amplification, samples were heated at 72°C for 15 min, and held at 4°C until needed. PCR fragments were sequenced when fidelity of the *Taq* DNA polymerase was crucial (e.g., when making expression constructs).

2.17 - RT-PCR

Each RT reaction contained 1 $\times$ Superscript RT buffer (supplied by manufacturer), 10 mM DTT, 0.25 mM of each dNTP, RNA sample (typically $\leq$ 1 μ g), 5 pmol primer (the primer that annealed to the sense strand in the subsequent PCR reaction was often used), and 200 units Superscript Reverse Transcriptase in a total volume of 20 μ L. DEPC-treated water, RNA and primer were pipetted into a 0.5 mL plastic microcentrifuge tube, heated to 65°C for 5 min, and chilled on ice. Buffer, DTT and dNTP were then added and the mixture preheated to 50°C for ~2 min. Superscript was added and the incubation continued at 50°C

for 1 h. PCR was then performed as described in section 2.16 except that the DNA template was substituted with $\leq 5 \mu\text{L}$ of RT reaction.

2.18 - Stock solutions

If ribonuclease contamination was a concern, solutions were mixed with DEPC (50 μL DEPC per 100 mL solution, room temperature, few hours) and then autoclaved for 30 min at 121 °C. Human GM-CSF (lyophilisate for human injection, a generous gift from Dr. Harold Atkins of the Cancer Research Group, Ottawa Regional Cancer Centre) was dissolved in sterile PBS at a concentration of 500 $\mu\text{g/mL}$, and stored frozen in aliquots at -100 °C. Repeated freezing and thawing of aliquots was avoided. Immediately before use, stock GM-CSF was diluted appropriately into sterile PBS or media. Actinomycin D and α -amanitin were dissolved in DMSO at 5 mg/mL and 6.25 mg/mL respectively, and stored in the dark at -20 °C. Cycloheximide was dissolved in sterile PBS at 10 mg/mL and stored at -20 °C.

Chapter 3

***In vitro* response of peripheral blood human granulocytes to human
GM-CSF**

3.1 - Introduction

Although GM-CSF was originally cloned for its ability to stimulate the formation of granulocyte and macrophage colonies in human bone marrow cultures [137,139], it also stimulates mature granulocytes via high affinity cell-surface receptors [71,183]. The effects of GM-CSF on human neutrophils are numerous and have been reviewed in greater detail in Chapter 1. With the exception of Chapter 6, the experimentation in this thesis will concentrate on the effect of GM-CSF on granulocyte transcription and translation. However, before proceeding to pertinent experiments, I needed to first verify that granulocytes respond as expected to cytokine stimulation. Since the vast majority of cells were neutrophilic granulocytes, the terms "neutrophil" and "granulocyte" will be used interchangeably throughout this thesis.

This chapter describes properties of my experimental system and compares them to the work of others. It has already been shown in this laboratory that granulocytes were capable of undergoing a respiratory burst when treated with the PKC agonist, PMA, verifying the viability and antimicrobial activity of these cells [184]. In this chapter, four additional experiments are described to confirm that both the granulocytes and GM-CSF were functional. (i) The low rates of macromolecular synthesis in granulocytes was verified by comparing the uptake of $^{32}\text{P}_i$, [^3H]uridine and [^{35}S]methionine in these cells to that of cultured HL-60 cells. (ii) The known ability of GM-CSF to delay the onset of granulocyte apoptosis was assessed by both cell morphology and DNA fragmentation. (iii) The ability of GM-CSF to induce changes in granulocyte shape was verified. (iv) Northern blotting was used to verify that GM-CSF transiently upregulates *c-fos* mRNA in mature human

granulocytes.

3.2 - Materials and Methods

3.2.1 - Quantifying cell-shape changes in granulocytes in response to GM-CSF

Human granulocytes were cultured with or without 20 ng/mL GM-CSF for 2 h, and then harvested. Cells in suspension were fixed by adding an equal volume of 8% formaldehyde in PBS and standing for several hours at room temperature. Cells were pelleted by low-speed centrifugation and were suspended in PBS. At least 150 cells were counted under phase contrast microscopy at 200× magnification in a hemacytometer. Cells were scored as either 'spherical' or 'misshapen'. % 'misshapen' = (#'misshapen') / (#'misshapen' + #'spherical') × 100%.

All other methodologies are described in Chapter 2 or in the respective figure legend.

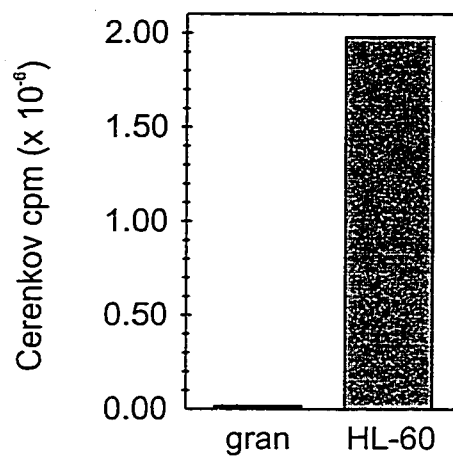
3.3 - Results

3.3.1 - Synthesis of macromolecules in mature granulocytes

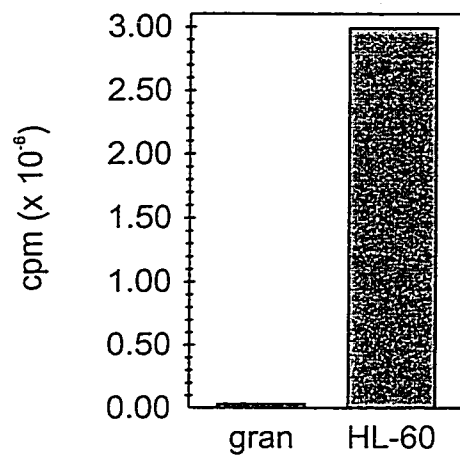
Mature granulocytes are considered to be terminally differentiated; they do not undergo cell division and they do not synthesize DNA. However, these cells synthesize RNA and protein at very low, but detectable, levels [1,38,171,185]. By quantifying the uptake of [³²P]orthophosphate (into organic phosphates such as nucleic acids and phospholipids), [³H]uridine (into RNA), and [³⁵S]Met (into protein), mature granulocytes were shown to incorporate 20- to 100-fold less radiolabel than cultured HL-60 cells (Figure 3.1). Note that, in the protein panel of Figure 3.1, radiolabeling times were longer for

Figure 3.1. Comparison of macromolecule synthesis between mature human granulocytes (gran) and promyelocytic HL-60 cells. *Organic phosphates.* 10^7 cells were radiolabeled for 45 min at 37°C with 50 μ Ci [32 P]orthophosphate in 10 mL 137 mM NaCl, 5 mM KCl, 0.8 mM MgSO_4 , 5 mM Glc, 0.5% (w/v) BSA, and 10 mM Hepes/NaOH (pH 7.4). Cells were pelleted by low-speed centrifugation, were washed extensively, and were counted by Cerenkov counting in a liquid scintillation counter (Liquid Scintillation Analyzer 1600TR, Canberra Packard Canada, Nepean, ON). Each bar is the mean of 3 determinations. *RNA.* 10^7 cells were radiolabeled for 6 h under standard tissue culture conditions with 50 μ Ci [5,6- 3 H]uridine in 10 mL media (HL-60, DMEM + 10 % FCS : gran, RPMI 1640 + 10% autologous serum). Total RNA was isolated from the radiolabeled cells as described in Chapter 2, and was counted in a liquid scintillation counter with scintillation fluid (Liquid Scintillation Cocktail R04679). Each bar depicts a single representative value. *Protein.* 5×10^6 cells were radiolabeled for 1.5 h (HL-60) or 4 h (gran) under standard tissue culture conditions with 25 μ Ci L-[35 S]methionine in 5 mL Met-free RPMI 1640 + 10 % serum. Cells were collected by low-speed centrifugation, and were washed extensively. Cell pellets were solubilized by heating in SDS sample buffer at 65-100°C, and were counter in a liquid scintillation counter with scintillation fluid. Each bar is the mean of 2 determinations.

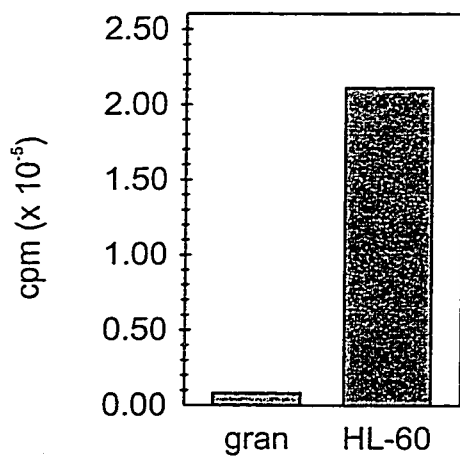
**ORGANIC
PHOSPHATES**



RNA



PROTEIN



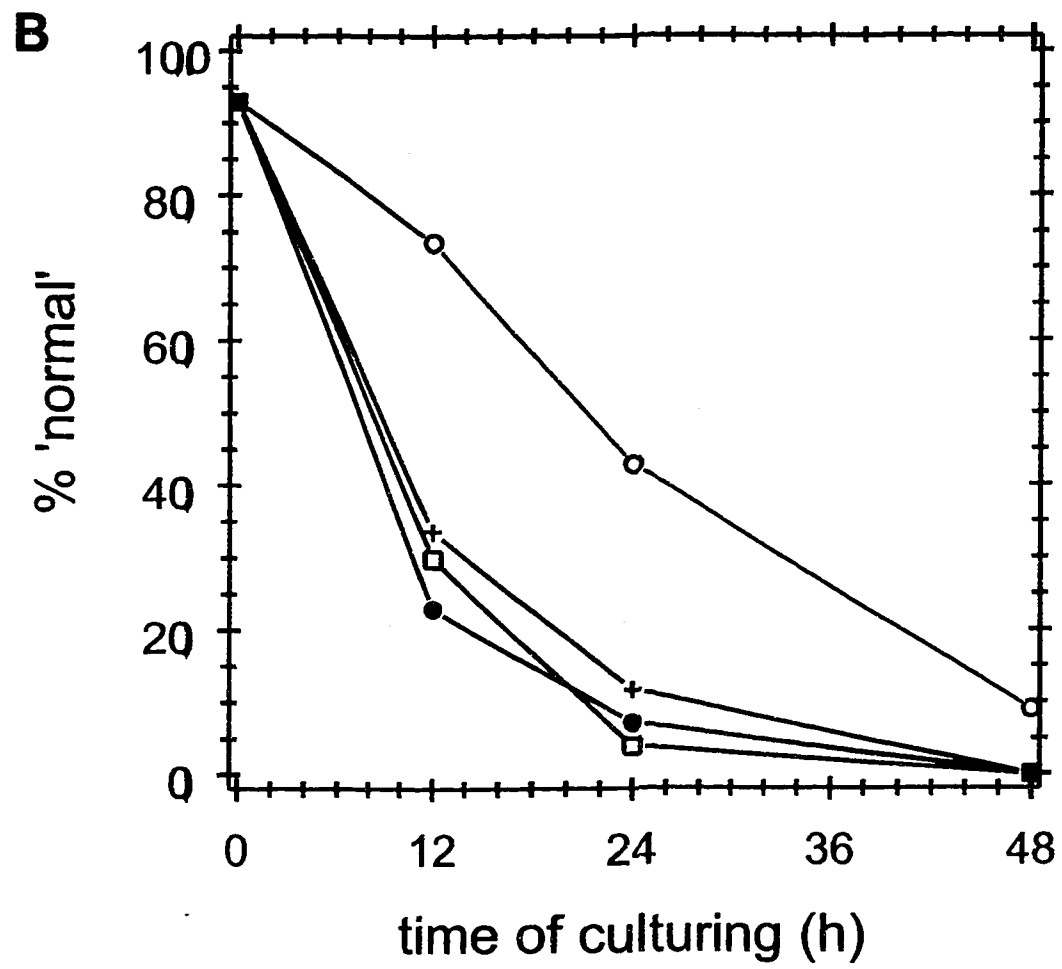
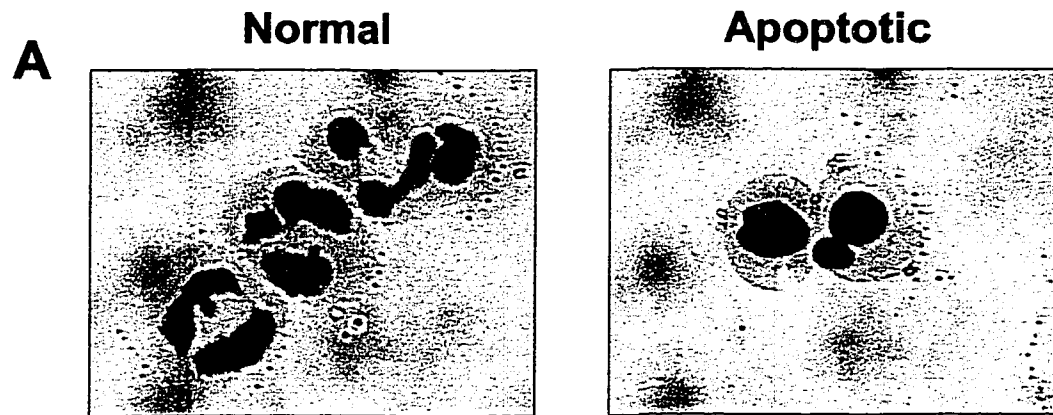
granulocytes than for HL-60 cells, but this does not affect the conclusions. The lower uptake of radiolabel into granulocytes is consistent with their low rates of macromolecule synthesis. However, differences in transport rates or metabolite pool sizes may also affect these pulse radiolabeling experiments.

Orthophosphate radiolabeling showed very little incorporation into granulocytes in Figure 3.1. However, in some earlier experiments, $^{32}\text{P}_i$ incorporation into granulocytes was equal to or greater than that of HL-60 cells (data not shown). This was eventually traced to a minor contamination of buffer solutions with *Pseudomonas fluorescens*, a common bacterial contaminant, as described in Appendix I. This low-level *P. fluorescens* contamination was avoided in all subsequent experiments by using strict aseptic techniques.

3.3.2 - Effect of GM-CSF on granulocyte apoptosis

Neutrophils undergo apoptosis spontaneously when cultured *in vitro*, where 50% of cells become apoptotic within 10-40 h [69-74]. The onset of apoptosis can be delayed by 6-32 h when cells are cultured with GM-CSF [69,71], although significantly longer delays have been reported [70]. The prolongation of their lifespan requires both RNA and protein synthesis [70]. Neutrophils develop a typical apoptotic morphology, including reduced cell volume, cytoplasmic vacuoles, and intensely heterochromatic, rounded nuclei [68,78]. Because of the unusual segmented appearance of 'normal' neutrophil nuclei, these morphological changes are easily scored under the microscope (see examples in Figure 3.2A). Blind assessment of the morphology of cultured neutrophils revealed that the cells were 50% apoptotic by ~10 h (Figure 3.2B, +). Culturing the cells with GM-CSF increased

Figure 3.2. GM-CSF delays apoptosis in cultured human granulocytes - assessment by cell morphology. Human granulocytes were cultured in 6-well tissue culture plates at 2×10^6 cells per well. After the indicated amount of time, 50 μ L aliquots were removed and cytopun onto glass slides ($400 \times g$, 5 min, high acceleration). Slides were fixed in methanol at room temperature for 5 min, and were stained with Diff-Quik according to the manufacturer's instructions. Slides were allowed to air-dry. Cell morphology was assessed by bright-field microscopy ($400\times$ magnification). At least 100 cells were scored blindly as either 'normal' or 'apoptotic' (see examples in *panel A* at $1000\times$ magnification). % 'normal' cells is plotted versus time of culturing in *panel B*. +, no addition; o, 20 ng/mL GM-CSF; ●, 20 ng/mL GM-CSF and 2 μ g/mL actinomycin D; □, 20 ng/mL GM-CSF and 10 μ g/mL cycloheximide. Plotted points are the means of 2 values derived from one donor.



this time to ~22 h (Figure 3.2B, ○). Inclusion of the transcription inhibitor, actinomycin D, or the translation inhibitor, cycloheximide, completely abrogated the delay induced by GM-CSF (Figure 3.2B, ● and □). Significant disintegration of apoptotic neutrophils occurred after 24 h of culture, especially with 'no addition', 'actinomycin D' and 'cycloheximide' samples (data not shown). Similar observations have been reported previously [68].

A hallmark biochemical feature of programmed cell death is the degradation of genomic DNA into nucleosome-ladders of ~200 bp [186]. Such DNA fragmentation was observed in neutrophils that had been cultured for 24 h (Figure 3.3A and B, compare 'No' lanes). The DNA from neutrophils stimulated with GM-CSF for 24 h was of higher average molecular weight, indicating that fewer cells were apoptotic (Figure 3.3A and B, lane GM). Inhibition of either transcription (Figure 3.3A and B, lanes Act and α Am) or translation (Figure 3.3A, lane Cyc) abrogated the GM-CSF-induced delay. α -Amanitin inhibits RNA polymerase II more selectively than actinomycin D [187], and was used in Figure 3.3 to demonstrate the dependence of the apoptotic delay on mRNA synthesis. I conclude that GM-CSF produced the expected delay in spontaneous apoptosis of mature neutrophils as assessed by both cellular morphology and DNA laddering, in agreement with the reports of others.

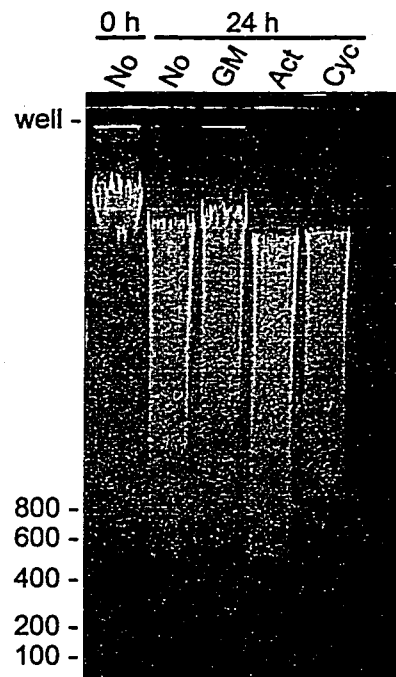
3.3.3 - Effect of GM-CSF on granulocyte shape

Within 5 min of its addition, 2 ng/mL GM-CSF induces human neutrophils to polarize and form irregular, elongated shapes [71]. These shape-changes are believed to be caused by membrane ruffling and random (i.e., nondirected) migration due to the activation

Figure 3.3. GM-CSF delays apoptosis in cultured human granulocytes - assessment by DNA degradation. Human granulocytes were cultured in 10-cm tissue culture plates at 10^7 cells per plate under the following conditions: No, no addition; GM, 20 ng/mL GM-CSF; Act, 20 ng/mL GM-CSF and 2 μ g/mL actinomycin D; Cyc, 20 ng/mL GM-CSF and 10 μ g/mL cycloheximide; α Am, 20 ng/mL GM-CSF and 5 μ g/mL α -amanitin. After the indicated amount of time (in h), genomic DNA was isolated from the cells as described in Chapter 2. Three μ g DNA was electrophoresed per lane on an agarose gel with 1 mM CDTA, 20 mM sodium acetate, 40 mM Tris/acetic acid (pH 7.8) as electrophoresis buffer. The agarose gel was stained, post-electrophoresis, with 1 μ g/mL ethidium bromide in water for 20 min. The stained gel was photographed under UV transillumination with a UVP ImageStore 7500 documentation system (DiaMed Lab Supplies Inc., Mississauga, Ontario). *Panels A and B* show the results from 2 different donors. DNA size markers (in bp) are indicated at the left of each panel.

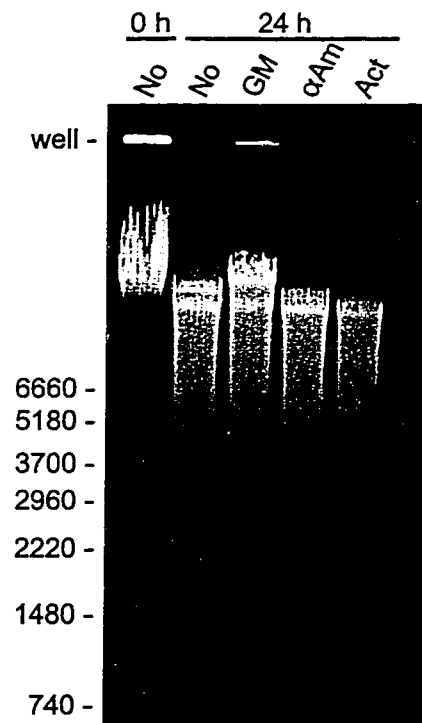
A

1.2 % agarose



B

0.8 % agarose



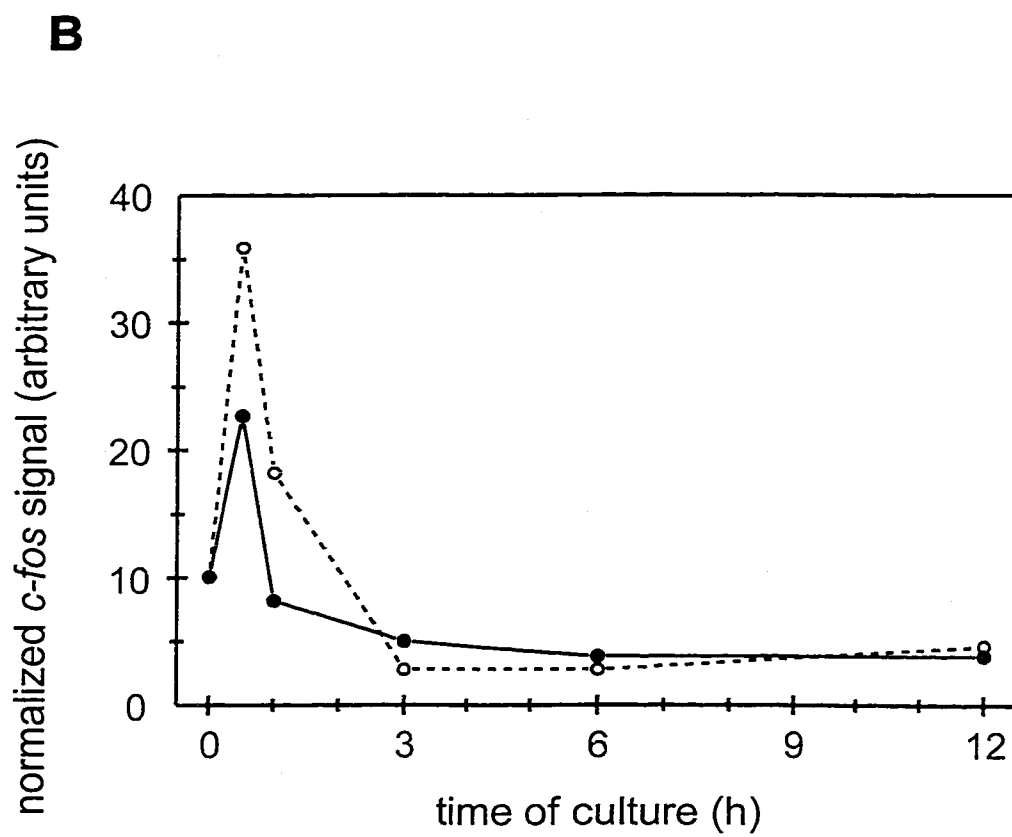
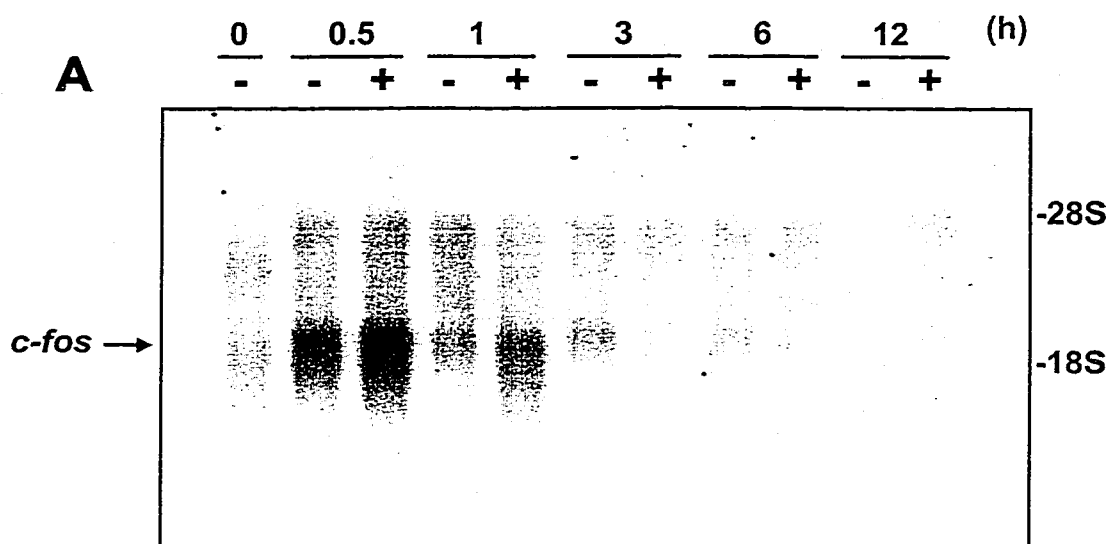
of myosin light chain kinases [154]. After 2 h of treatment with 20 ng/mL GM-CSF, $89.3 \pm 1.9\%$ (mean $\pm$ SEM, $n = 3$) of cultured human granulocytes were 'misshapen' compared to $22.3 \pm 2.7\%$ (mean $\pm$ SEM, $n = 3$) without cytokine treatment. In addition to the 4-fold increase in number of 'misshapen' cells, GM-CSF also augmented the severity of the polarization in individual cells (data not shown). Therefore, cytokine stimulation did induce cell polarization as anticipated. The reason that ~20% of untreated cells also demonstrated shape-changes will be discussed later. Because monitoring was rapid and simple, shape-changes were routinely used to verify that each aliquot of GM-CSF was biologically active.

3.3.4 - Effect of GM-CSF on *c-fos* mRNA levels in granulocytes

The protooncogene and transcription factor *c-fos* is upregulated transiently by growth factors in a wide range of cell-types [188-191]. The mRNA has a high rate of turnover due to AU-rich sequences in its 3'-UTR and has a typical half-life of only 6 to 9 min [148,192,193]. Interestingly, mature human granulocytes contain appreciable levels of *c-fos* mRNA [14,15,194], although it has been reported that transcript levels become undetectable after 2 h of *in vitro* incubation [195]. Various stimulators of granulocyte function, including GM-CSF, increase *c-fos* levels [15,195]. To my knowledge there is no published time-course of *c-fos* induction by GM-CSF in these cells; however, the upregulation of *c-fos* induced by the chemotactic peptide fMLP was shown to peak at 15 min and then become undetectable by 2 h [195]. In the example shown in Figure 3.4, freshly isolated granulocytes (0 time-point) possessed a relatively low, but detectable *c-fos* signal at ~2.2 kb. Granulocytes cultured without GM-CSF (Figure 3.4A, - lanes, and Figure 3.4B, ●) showed

Figure 3.4. **Time-course of transient *c-fos* mRNA induction in cultured human granulocytes.** All procedures are outlined in Chapter 2. Part A. Northern blot for *c-fos*. Human granulocytes were cultured with (+) or without (-) 1 ng/mL GM-CSF, and total RNA was isolated from the cells after the indicated amount of time (h). Total RNA equivalent to 1.5×10^7 cells ($\sim 3 \mu\text{g}$) was electrophoresed per lane of a formaldehyde agarose gel and was transferred by vacuum to a nylon membrane. The membrane was probed with the random-primed 1.3 kbp *Pst*I fragment of *pfos-1* [196]. Shown is the Northern blot, visualized on a PhosphorImager screen. The positions of 28S rRNA, 18S rRNA and *c-fos* are highlighted. Part B. Quantitation of Northern blot shown in part A. The intensity of the *c-fos* signal was quantified by PhosphorImager technology, and the signals normalized with a human 28S rRNA probe. Shown is a plot of the normalized signal intensity versus time of cell culture. ● *solid line*, cultured without GM-CSF; ○ *dashed line*, cultured with 1 ng/mL GM-CSF.

-47-
Figure 3.4



an increased *c-fos* signal that was reduced to baseline levels within 1 h. The cell isolation and/or *in vitro* culturing may have triggered this response. Stimulation with 1 ng/mL GM-CSF (Figure 3.4A, + lanes, and Figure 3.4B, ○) caused a ~2-fold increase in *c-fos* levels at 30 min compared to unstimulated cells. This elevated signal persisted to 60 min and was reduced to baseline levels by 3 h. Thus, the time-course of *c-fos* upregulation seen with GM-CSF was comparable to that reported for fMLP [195]. In both stimulated and unstimulated samples in Figure 3.4, a low signal was present near the expected position of *c-fos* from 3 to 12 h. Distinguishing continued low expression of the transcript from cross-reaction with 18S rRNA is difficult with the technique used. Some cross reaction with 28S rRNA appears to be present also.

3.4 - Discussion

Several properties of human peripheral blood granulocytes isolated in our laboratory are similar to those described by others. These include low rates of macromolecular synthesis, spontaneous apoptosis when cultured *in vitro* and detectable levels of *c-fos* mRNA. In addition, GM-CSF stimulation delayed the onset of apoptosis, increased the level of *c-fos* mRNA for at least 1 h and induced changes in cell shape. A mature neutrophil expresses about 540 GM-CSF receptors on its cell-surface with a K_d of 20-100 pM or 0.4-2 ng/mL [143,145]. Except for three cases (i.e., Chapter 3, Figure 3.4, 1 ng/mL; Chapter 4, Figure 4.6B, 0.01-10 ng/mL; and Chapter 4, Figure 4.7, 1 ng/mL), the concentration of GM-CSF used in this thesis was 20 ng/mL, which is at least 10× greater than the K_d and should saturate >90% of the receptors. However, GM-CSF concentrations as low as 0.1 ng/mL have

also been shown to delay the onset of apoptosis [69] and to induce cell polarization [71]. Although the effect of GM-CSF on neutrophil populations was studied throughout this thesis, responses are known to vary considerably from cell to cell. This was demonstrated in Chapter 4 using cellular autoradiograms of [³H]uridine-labeled neutrophils. The reason for such variability is not known, but could be related to the age of the cell; 'older' neutrophils in the population may be less responsive to the cytokine than 'younger' neutrophils.

Stimulation of cultured neutrophils with GM-CSF produced the expected delay in the onset of apoptosis. I observed ~10 h to be the median time of onset of apoptosis in unstimulated cells, which is at the lower limit of published values (i.e., 10-40 h). The median times of onset of spontaneous apoptosis and of GM-CSF-induced apoptotic delay vary widely between reports [69-74]. The reason for such variability is not clear, but could be caused by one or more of the following factors. (i) Donor variability is an important consideration. The data from Figure 3.2 were derived from one normal donor whose cells may have had a shorter than average lifespan. However, many published reports have used values averaged from multiple donors, and yet variability still exists. (ii) Granulocyte culturing conditions vary widely, including variations in cell concentration, use of antibiotics, types of media, serum and culturing vessels. For example, IL-6 has been shown to delay neutrophil apoptosis at high cell concentrations of $1-2 \times 10^7/\text{mL}$ but not at lower cell concentrations of $1-5 \times 10^6/\text{mL}$ [115]. Autocrine activity of PAF induced by IL-6 has been implicated in this particular example [116]. (iii) The concentration of GM-CSF used by different workers varies and this could have had an effect. Cox *et al.* [69] and Lee *et al.* [74]

found that the degree of apoptotic delay induced by GM-CSF was indeed directly proportional to the concentration of the cytokine. (iv) The technique used to isolate the neutrophils should also be taken into consideration. Many variations have been used in the literature, most involving density gradient centrifugation and lysis of contaminating erythrocytes with NH_4Cl or water. NH_4Cl is a membrane-permeable weak base that causes transient changes in intracellular pH [197]. NH_4Cl was shown to inhibit degranulation of human neutrophils [198], although other processes such as shape changes and superoxide anion release following LPS stimulation appeared unaffected [199]. Also, exposure to Ficoll-Hypaque has been shown to affect some neutrophil functions, including the induction of spontaneous cell polarization [199]. Haslett *et al.* reported that 30.5% of neutrophils exhibited spontaneous change of shape when isolated with a Ficoll-Hypaque procedure [199]. This likely explains why our neutrophil preparations were 22.3% 'misshapen' when cultured without GM-CSF. The procedure used to isolate granulocytes in this thesis afforded a high recovery of relatively pure cells that responded appropriately to the tests in this chapter.

HL-60 cells are a well-characterized human cell-line, considered to be of promyelocytic origin. These cells are precursors to mature neutrophils and can be differentiated to granulocyte-like cells with various agents (see Chapter 1, section 1.2). As apparent in Figure 3.1, the very low transcription and translation rates characteristic of mature neutrophils have not yet manifested themselves at the promyelocyte stage of differentiation. Torelli *et al.* found that the sequence complexity of total poly(A)⁺ RNA dropped from 64,000 to 26,000 different sequences when HL-60 promyelocytes were

differentiated to granulocytic cells with all-*trans* RA [200]. This reduction in synthesis of mature mRNA may be necessary for neutrophil differentiation or may be the result of the phenotype of mature neutrophils (i.e., their deficiency of many common subcellular structures). This hypothesis will be addressed in more detail in Chapter 4. Chapter 4 will concentrate on transcription in mature human granulocytes with and without GM-CSF stimulation. The goal of this part of my work was to identify novel transcripts and/or novel aspects of transcription in these unusual cells.

Chapter 4

RNA metabolism and novel transcripts in human granulocytes

4.1 - Summary

Two approaches were undertaken to study the RNA synthesized in mature granulocytes in response to GM-CSF: (i) [^3H]uridine radiolabeling to analyze overall RNA synthesis and (ii) DDRT-PCR to identify specific, differentially expressed transcripts. Stimulation of granulocytes with the cytokine caused an increase in [^3H]uridine incorporation by as much as 10-fold in 6 h. At least 90% of the radiolabeled RNA was derived from polymerase II transcription with the remaining ~10% resembling mature tRNA. There was no evidence of ribosomal RNA synthesis. Despite the propensity toward polymerase II transcription in a 6 h pulse, only 12-22% of the ^3H -RNA was found in the cytoplasm and only ~25% bound to oligo(dT) cellulose. This suggests that most of the radiolabel was nonpolyadenylated hnRNA.

DDRT-PCR of granulocyte total RNA identified several transcripts, 2 of which were studied further. (i) Human *sgk* was upregulated within 30 min of stimulation with ≥ 0.1 ng/mL GM-CSF. mRNA levels peaked at 1-3 h (~10-fold increase) but remained elevated for at least 12 h. The function of this putative kinase in granulocytes and other cell types is not known currently. (ii) Human serglycin intron 2 was amplified from *hsgly* hnRNA and could be detected readily on granulocyte Northern blots. GM-CSF had no significant effect on the expression level of this transcript. Granulocyte Northern blots were then probed with sequences directed against *hsgly* introns 1 and 2. Both intron signals were found in greater quantities in granulocytes compared to HL-60 cells, but the normalized intron 1 signal appeared higher. A model is proposed in which mature granulocytes are slow to elongate polymerase II transcripts in the nucleus. This model is supported by both the radiolabeling

and *hsgly* data. However, more experiments are required to determine if this property of the granulocyte *hsgly* gene is also shared by other transcripts expressed in the mature granulocyte.

4.2 - Introduction

4.2.1 - RNA metabolism in mature granulocytes

Mature human granulocytes possess very low rates of RNA synthesis as measured by incorporation of [³H]uridine (e.g., see Figure 3.1). Low transcription rates in the mature neutrophil are associated with loss of subcellular structures such as mitochondria and ER. Although [³H]uridine incorporation is ~100-fold lower than in promyelocytic HL-60 cells, stimulation with a variety of proinflammatory agonists such as GM-CSF, TNF α , fMLP, or IL-13 [201,202] causes an increase in radiolabeling (typically <5-fold), which is indicative of some residual transcriptional capacity in the neutrophil. Electron micrographs of these cells reveal no visible nucleoli and few ribosomes, suggesting that rRNA synthesis has slowed or ceased [1,25,38]. When total RNA from [5-³H]uridine-labeled granulocytes was examined by gel electrophoresis, Beaulieu *et al.* found no radiolabeled 28S or 18S rRNA [201]. There was little discussion of the significance of this observation. Granelli-Piperno *et al.* found that most radioactivity was localized over the nucleus in cellular autoradiograms of human neutrophils that had been labeled with [³H]uridine for 4 h [171]. No further experimentation was performed to explain this observation.

Despite the low rate of RNA synthesis, mature granulocytes are known to synthesize some mRNA constitutively, including *c-raf* [203], *c-fos* [194], IL-1Ra [159], 5-lipoxygenase

[157], CR1, actin, MHC class I and CR3- β [204]. In addition, granulocytes can be stimulated to transcribe certain mRNA. Examples include *c-fos* and IL-1Ra in response to TNF α [15,159], FLAP, *c-fos*, IL-3 receptor α , 5-lipoxygenase, IL-8 and IL-1Ra in response to GM-CSF [15,155-159], IFN- α and *c-fos* in response to G-CSF [15,205], MIP-1 α , IL-8 and IL-12 p40 in response to LPS [206-208], and *c-fos* in response to fMLP [195]. That GM-CSF can induce the synthesis of many species of mRNA is not surprising since [3 H]uridine incorporation is stimulated ~3-fold after 4 h [201]. Many of the transcripts that have been identified as GM-CSF-responsive are involved in the neutrophilic inflammatory response. Much emphasis has been placed on such mRNA because of the highly specialized functions of phagocytic leukocytes. However, granulocytes do transcribe more than just proinflammatory agonists and antibacterial products. For example, actinomycin D, α -amanitin or cycloheximide can abrogate the apoptotic delay induced by GM-CSF-treatment, suggesting the continued transcription and translation of some unknown anti-apoptotic molecule(s) (see Chapter 3). The identification of inducible, 'noninflammatory' transcripts is a major subject of this chapter.

4.2.2 - Serum- and glucocorticoid-regulated kinase (*hsgk*)

Protein kinases are often regulated post-translationally by protein interactions or by phosphorylation. However, there is a subfamily of serine/threonine protein kinases that are regulated transcriptionally. This subfamily includes *snk* (serum-inducible kinase) [209], *plk* (polo-like kinase) [210-213], *fnk* (FGF-inducible kinase) [214], and *sgk* (serum- and glucocorticoid-regulated kinase) [215,216]. *Sgk* was originally cloned as a glucocorticoid-

inducible transcript in Con8.hd6 rat mammary tumour cells [215]. The 2.4 kb *sgk* transcript encoded a protein of 49 kDa. The deduced amino acid sequence shared significant identity (45 to 55%) throughout its putative catalytic domain with other serine/threonine protein kinases including PKC, PKA, rac protein kinase and ribosomal protein S6 kinase. However, *sgk* kinase activity has not yet been confirmed experimentally. Elevated rodent *sgk* mRNA expression has also been demonstrated in epithelial cells and fibroblasts in response to serum [215,217], in ovarian granulosa cells in response to follicle-stimulating hormone and testosterone [218], in oligodendrocytes in response to cortical injury [219], and in glial cells in response to excitotoxic hippocampal lesions [220]. Suppression of *sgk* expression by heparin has been shown in proliferating vascular smooth muscle cells of rat [221].

Human *sgk* (*hsgk*) was recently cloned and shown to have 98% sequence identity to that of the rat. The 2.6 kb *hsgk* transcript was strongly induced in a human hepatoma cell line during hypertonic treatment, although the significance of hypertonic upregulation is not clear [216]. This induction occurred independently of *de novo* protein synthesis and could be maintained for at least 8 h. The human transcript was not induced by dexamethasone treatment, suggesting a transcriptional control distinct from that of the rat. Although Northern blots revealed a broad tissue distribution, *sgk* expression has not previously been demonstrated in mature human granulocytes [216].

4.2.3 - Serglycin (*hsgly*)

Serglycin (also called HpPG) is the peptide core of proteoglycans found in the secretory granules of hematopoietic cells [222-224]. This small protein (mw = 17.6 kDa in

human) contains a protease resistant, 18-amino acid region of alternating serine and glycine residues, from which its name was derived [222,223]. Attached to the serine residues are O-linked glycosaminoglycans such as heparin, heparan sulfate, and various chondroitin sulfate derivatives [224]. The presence of a high density of sulfated sugars gives the serglycin proteoglycan a strong negative charge. The proteoglycans associate tightly via ionic interactions with basic proteins found in hematopoietic secretory granules. The serglycin proteoglycans are believed to serve several functions, including suppression of hydrolytic enzyme activity during storage, retention of granule constituents at the cell-surface after secretion, and facilitation of the packaging and sorting of secretory proteins [7,225]. Serglycin proteoglycans are also ligands for the lymphocyte adhesion molecule CD44 [226].

The 16.7 kb human serglycin gene contains 3 exons and 2 large introns, and is encoded on chromosome 10q22.1 [224]. The ~1.3 kb mRNA is expressed primarily in hematopoietic cells, including promyelocytic HL-60, mature neutrophils, basophils, eosinophils, natural killer cells, megakaryocytes, and mast cells [222,227,228]. Differentiation of HL-60 to granulocyte-like cells by RA, arachidonic acid, or eicosapentaenoic acid treatment has been shown to reduce serglycin mRNA up to 10-fold by 2 d [229], although Stellrecht *et al.* found no change after 5 d with RA or after 3 d with DMSO [230]. The reason for this discrepancy has not been addressed. However, serglycin mRNA levels increase as pluripotent stem cells differentiate to promyelocytes, and gradually decrease as the cells mature further; *in situ* hybridization of the HpPG cDNA to normal bone marrow revealed a 2-fold decrease in expression as promyelocytic cells matured [227]. This is believed to reflect cellular demands for serglycin protein since granule synthesis peaks

near the promyelocyte/myelocyte stage but decreases thereafter [7].

This chapter describes the study of RNA transcription in human granulocytes cultured in the absence or presence of GM-CSF. An analysis of ^3H -RNA synthesized with GM-CSF-treatment was performed, identifying the type of RNA (i.e., mRNA, rRNA or tRNA) and its cellular localization. DDRT-PCR was then used to identify novel transcripts. Two of these species were characterized further: (i) *hsgk* mRNA, which is upregulated 10-fold by GM-CSF stimulation and (ii) *hsgly* hnRNA, which can be detected readily on Northern blots but is not affected by GM-CSF. The significance of my findings will be discussed.

4.3 - Materials and Methods

4.3.1 - Fractionation of nuclear and cytoplasmic RNA using nonionic detergent

A washed pellet of human granulocytes was gently suspended in ice-cold lysis buffer (140 mM NaCl, 1.5 mM MgCl_2 , 0.5 % (v/v) NP-40, 20 mM ribonucleoside-vanadyl complexes and 10 mM Tris/HCl (pH 8.6) : 1-2 μL per 10^5 cells). The mixture was vortexed for 10-20 s, allowed to stand on ice for 5 min, and centrifuged at $500\times g$ for 5 min at 2°C . The supernatant (cytoplasmic RNA) was diluted into 4 mL RES (1 M urea, 1% SDS, 0.5 M LiCl, 5 mM CDTA, 100 $\mu\text{g/mL}$ proteinase K and 40 mM Mops/NaOH (pH 6.8)). The pellet (nuclear RNA) was re-extracted with ice-cold lysis buffer. The resulting supernatant was pooled with that above. The pellet was suspended in lysis buffer, and diluted into 4 mL RES. Both RES samples (i.e., cytoplasmic and nuclear RNA) were lightly sonicated, and then digested at 37°C for 30 min. Glycogen (40 μg) was added to the cytoplasmic RNA as

a carrier. Both RES samples were precipitated with 2 volumes ethanol/0.1 vol. sodium acetate (pH 5.6) overnight at -20°C . The precipitates were collected by centrifugation in a swing-out bucket rotor (10,000 rpm, 0°C , 30 min, Sorvall HB-6 rotor), washed twice with 80% ethanol, and allowed to air-dry. Subsequent procedures depended on the type of analysis being performed.

(i) Quantifying radiolabeled RNA

The dried pellets were dissolved in RES and counted in a liquid scintillation counter with scintillation fluid.

(ii) Quantifying RNA mass

The dried pellets were dissolved in 300 μL RES and were digested for 30 min at 37°C . The samples were extracted with 50 μL phenol/chloroform (1 g phenol per mL CHCl_3), and back-extracted with 100 μL RES without protease. The pooled aqueous phase was extracted once with CHCl_3 . An equal volume (i.e., 400 μL) of 3 M LiCl/40 % (v/v) ethanol was added to the aqueous phase, and the RNA allowed to precipitate overnight in ice-water. The sample was centrifuged at room temperature for 4 min at $13,000 \times g$, and the pellet dissolved in 100 μL 0.1% SDS, 1 mM CDTA, 10 mM sodium phosphate (pH 6.8). The sample was precipitated a second time with an equal volume of 3 M LiCl/40 % ethanol as above. The resulting pellet was dissolved in 50 μL 0.1% SDS, 1 mM CDTA, 10 mM sodium phosphate (pH 6.8), and was precipitated with 2 volumes ethanol and 0.1 volume 3 M sodium acetate (pH 5.6) at -20°C for ≥ 20 min. RNA was pelleted by centrifuging at $13,000 \times g$ for 4 min at room temperature. The pellet was washed with 70 % ethanol, dried under vacuum, dissolved in 400 μL DEPC-treated water, and quantified by absorbance at

260 nm.

4.3.2 - Fractionation of nuclear and cytoplasmic RNA using hot phenol at pH 5.1 [231]

1×10^7 human granulocytes were radiolabeled with 50 μCi [5,6- ^3H]uridine for 5 h with 20 ng/mL GM-CSF under standard culturing conditions. The cells were harvested, washed with PBS, suspended in 40 μL cold PBS, and transferred to a glass 13 $\times$ 100 mm screw-cap culture tube. To the cell suspension was added 1.96 mL cold 50 mM sodium acetate, 10 mM EDTA (pH 5.1), followed by 2 mL of phenol (saturated with 1 mM CDTA, 10 mM Tris/HCl (pH 7.5)). The mixture was shaken gently for 3-4 min at 60°C, chilled on ice, and centrifuged at room temperature for 10 min at 2000 rpm. The lower phase (i.e., phenol) was removed with a Pasteur pipette. The remaining aqueous phase (and interphase material) was extracted 2 $\times$ with fresh phenol as described above. A portion of the pooled phenol phase was diluted into scintillation fluid. The interphase material was dissolved in 1 N NaOH and diluted into scintillation fluid. The aqueous phase was precipitated overnight at -20°C with 100 μg carrier tRNA/0.1 volume sodium acetate (pH 5.6)/ 2 volumes ethanol. The RNA was pelleted by centrifugation at 10,000 rpm for 10 min at 4°C. The pellet was washed with 70% ethanol, dissolved in 0.1 N NaOH, and diluted into scintillation fluid. The radioactivity in the interphase, phenol and aqueous samples was quantified by liquid scintillation counting.

4.3.3 - Cellular autoradiography of radiolabeled granulocytes

5×10^6 human granulocytes were radiolabeled with 25 μCi [5,6- ^3H]uridine and 20

ng/mL GM-CSF for 6 h under standard culturing conditions. The cells were harvested, washed with PBS, and suspended in 2 mL ice-cold PBS. Fifty μ L aliquots were cytopspun onto polylysine-coated microscope slides (400 $\times$ rpm, 5 min, high acceleration), and the slides allowed to air-dry. The slides were then treated sequentially with the following solutions at room temperature. (i) methanol, 5 min (ii) 4 % formaldehyde in PBS, 10 min (iii) PBS, 5min (iv) twice with water, 5min each. The slides were allowed to air-dry. Slides were dipped individually into autoradiographic emulsion (Hypercoat LM-1), excess emulsion was allowed to drain, and the slides left to dry for 4 h. Dried slides were transferred to light-tight, desiccated slide-boxes and were stored for 6 weeks at 4°C. Boxes were allowed to equilibrate at room temperature for 1 h. Slides were placed in Kodak Dektol developer for 2 min, water for 30 s, 30% (w/v) sodium thiosulfate for 10 min, and gently running tap water for 20 min. All procedures involving autoradiographic emulsion were performed in total darkness until treatment with thiosulfate. Slides were allowed to air-dry completely. Nuclei were counterstained in 0.1% methylene blue for 60 min. Destaining was achieved by soaking slides in water for 30 min, 70% ethanol for 18 h, and then 100% ethanol for 30 min. Slides were allowed to air-dry completely, and were mounted with a coverslip and Permount mounting media.

4.3.4 - Binding of radiolabeled RNA to oligo(dT) cellulose

1×10^7 human granulocytes were radiolabeled with 50 μ Ci [5,6- 3 H]uridine for 6 h with 20 ng/mL GM-CSF under standard culturing conditions. Total RNA was isolated from the cells by the guanidinium method as described in Chapter 2. Oligo(dT) cellulose was

prewashed sequentially as follows. (i) once with 0.1 N NaOH, (ii) twice with DEPC-treated water, and (iii) twice with binding buffer (0.5 M LiCl, 0.1% SDS, 1 mM CDTA, 10 mM Tris/HCl (pH 7.5)). The washed oligo(dT) cellulose was suspended in binding buffer at 60-80 mg/mL. An aliquot of ^3H -RNA (typically $\sim\frac{1}{4}$ of the total sample) was diluted to 45 μL with DEPC-treated water, heated to 65°C for 5 min, and chilled on ice. The sample was transferred to a 1.5 mL screw-cap polypropylene microcentrifuge tube already containing 100 μL oligo(dT) suspension, 50 μL binding buffer and 5 μL 5 M LiCl. The sample was mixed on a rotary mixer (room temperature, 30 rpm) for 20-30 min. The sample was microcentrifuged for 10 s and the supernatant carefully removed. The pellet of oligo(dT) cellulose was washed $2 \times 200 \mu\text{L}$ with room temperature binding buffer by gentle inversion, then $3 \times 200 \mu\text{L}$ with eluting buffer (2 mM CDTA, 0.1% SDS). The pooled binding buffers (600 μL , nonbound fraction) and pooled eluting buffers (600 μL , bound fraction) were counted in a liquid scintillation counter with scintillation fluid.

4.3.5 - Differential display RT-PCR of granulocyte total RNA

The method of DDRT-PCR was performed similar to that originally described by Liang and Pardee [232,233]. Total RNA was isolated from granulocytes that had been cultured for 2 h either with or without 20 ng/mL GM-CSF. Any contaminating DNA was removed from the RNA samples by digesting with RNase-free DNase I (0.15 unit/ μL with 0.2 unit/ μL placental ribonuclease inhibitor) for 30 min at 37°C. The samples were deproteinized by phenol-chloroform extraction, concentrated by ethanol-precipitation, and dissolved in 20 μL 1 mM CDTA, 10 mM Tris/HCl (pH 7.5). RNA concentration was

estimated by its absorbance at 260 nm. Reverse transcription was carried out as follows. DNA-free total RNA (0.2 µg per reaction) was diluted into reverse transcriptase buffer containing 10 mM DTT, 20 µM of each of the 4 dNTP and 1 µM degenerate anchored oligo(dT) primer (either primer 4.1, 4.2 or 4.3 from Table 4.I) in a total volume of 19 µL. Samples were heated to 65°C for 5 min and were preincubated at 37°C for 10 min. Superscript II reverse transcriptase was added (200 units per reaction), the incubation continued at 37°C for 50 min, and the reaction heat-inactivated for 5 min at 95°C.

PCR was performed on RT samples as follows. Each PCR reaction (20 µL) contained 50 mM KCl, 0.05% Tween-20, 0.1 mg/mL BSA, 1.5 mM MgCl₂, 20 mM Tris/HCl (pH 8.5), 2 µM of each of the 4 dNTP, 0.1 µCi [α -³²P]dATP, 1 µM degenerate anchored oligo(dT) primer (same as used in RT reaction), 0.2 µM arbitrary primer (one of primers 4.4-4.10 from Table 4.I), 2 µL RT reaction and 1.5 unit *Taq* DNA polymerase. The reactions were overlayed with silicone oil, and amplified as follows: 40 cycles - 94°C / 30 s, 40°C / 2 min, 72°C / 30 s; 1 cycle - 72°C / 5 min; held at 4°C. Four µL of the PCR reaction was mixed with 2 µL loading buffer (10 mM CDTA, 1 mg/mL bromophenol blue, 1 mg/mL xylene cyanol in formamide) and was heated at 80°C for 2 min. All 6 µL was loaded on a polyacrylamide DNA sequencing gel (Bio-Rad 38 × 50 × 0.04 cm, 6% T:5% C containing 7 M urea and TBE as electrophoresis buffer, pre-run for 2 h at 80 W). Samples were electrophoresed at 80 W until the xylene cyanol had migrated 10 cm from the bottom of the gel (≥4 h). The unfixed gel was dried onto Whatman 3MM chromatography paper with a Bio-Rad gel dryer (50°C, 1 h, under vacuum), and was exposed to x-ray film for 24 h at room temperature. Using the film as a guide, differentially expressed bands were cut from

Table 4.I
Synthetic oligodeoxynucleotides used as primers in Chapter 4

Oligonucleotides were synthesized by Life Technologies (Burlington, Ontario) at a 50 nmole scale and standard purity. DDRT-PCR primers 4.4 to 4.10 were derived from those of Bauer *et al.* [234] with the addition of an *EcoRI* restriction site at the 5'-end.

<u>Primer</u> <u>ID #</u>	<u>Primer Length</u> <u>(bases)</u>	<u>PCR Annealing</u> <u>Temperature</u> <u>(°C)</u>	<u>Primer Sequence (5' – 3')</u>
4.1	18	40	AAGGCCTTTTTTTTTTTTG
4.2	18	40	AAGGCCTTTTTTTTTTTTC
4.3	18	40	AAGGCCTTTTTTTTTTTTA
4.4	15	40	CAGAATTCAACGAGG
4.5	15	40	CAGAATTCTGGCTCC
4.6	15	40	CAGAATTCGTCATAG
4.7	15	40	CAGAATTCCTTGATG
4.8	15	40	CAGAATTCCTAAGGC
4.9	15	40	CAGAATTCATACAGG
4.10	15	40	CAGAATTCCTCAGAC
4.11	21	58	ATTTCCTAATCAAGAAGTTGG
4.12	20	58	GGAGTTAATTTGGGGTGTTA
4.13	21	56	ATGCCCTGCCTTATCCTGTTT
4.14	25	56	TTCTGGGTTGAGTGTGATGATTAGA
4.15	21	51	AGAAAGAGACAAGGGAGAAAC
4.16	21	51	GAGGCAATAAAAAGTGGAAGAT
4.17	24	56	GAACTACTTCCAGGTGAATCCAAC
4.18	24	56	GAAGAAAAACACTCCCACATCTGT

the dried gel with a razor blade. Each gel slice was rehydrated in 100 μ L water, then boiled for 15 min. Fifty μ g glycogen was added to the supernatant, and the mixture precipitated with 4 volumes ethanol, 0.1 volume 3 M sodium acetate (pH 5.6) for 30 min at -70°C . The suspension was pelleted by microcentrifugation at 4°C for 10 min, washed with ethanol, air-dried, and dissolved in 10 μ L water. Four μ L was re-amplified using the PCR conditions above (same primer pair originally used) except that 20 μ M of each of the 4 dNTP was used, the PCR reaction volume was 40 μ L, and the radioisotope was omitted. The presence of a specific band after second PCR amplification was verified by electrophoresing an aliquot on 1.5% agarose and staining with ethidium bromide.

If the second PCR product was derived from both the anchored and arbitrary primers, it was digested with *Stu*I and *Eco*RI, and was ligated into *Sma*I / *Eco*RI-digested pBluescript KS(+) with T4 DNA ligase. If the second PCR product was derived from the anchored primer only, it was digested with *Stu*I, and was ligated into *Sma*I-digested and CIP-dephosphorylated pBluescript KS(+). Ligation mixtures were electroporated into competent XL-1 Blue, SURE or DH5 α *E. coli*. Bacteria were plated on LB agar + 100 μ g/mL ampicillin + X-gal (20 mg/mL in dimethylformamide, 30 μ L per plate) + IPTG (0.1 M, 30 μ L per plate). Plates were incubated at 37°C overnight. White colonies were inoculated into 2 mL 2 $\times$ YT containing 100 μ g/mL ampicillin and allowed to grow overnight at 37°C with vigorous shaking. Plasmid mini-preps were performed on portions of the cultures, and the presence of an insert verified by restriction endonuclease mapping. Clones of interest were sequenced using either a T7 or T3 promoter primer. To determine their identity, acquired sequences were submitted to NCBI BLAST nucleotide searches [235].

4.3.6 - Differentiation of HL-60 cells with RA

HL-60 cells were plated in 10-cm tissue culture plates at 5×10^5 cells/mL in 10 mL DMEM + 10% FCS. All *trans*-RA was added to 1 μ M and the cells allowed to incubate at 37°C in a humidified, 5% CO₂ atmosphere. After either 2 d or 4 d of culture, cells were harvested and total RNA was isolated as described in Chapter 2. RA differentiates HL-60 cells into granulocytic cells, which subsequently die via apoptosis (~10% apoptotic cells at day 4) [11]. Differentiation of our cells was verified by the appearance of apoptotic bodies under phase contrast microscopy after 4 d in culture. From the work of Martin *et al.* [11], approximately 20% and 50% of the cells were expected to be granulocytic after 3 and 5 d of culturing respectively. The remainder of the cells were expected to be promyelocytes, myelocytes, metamyelocytes and apoptotic bodies.

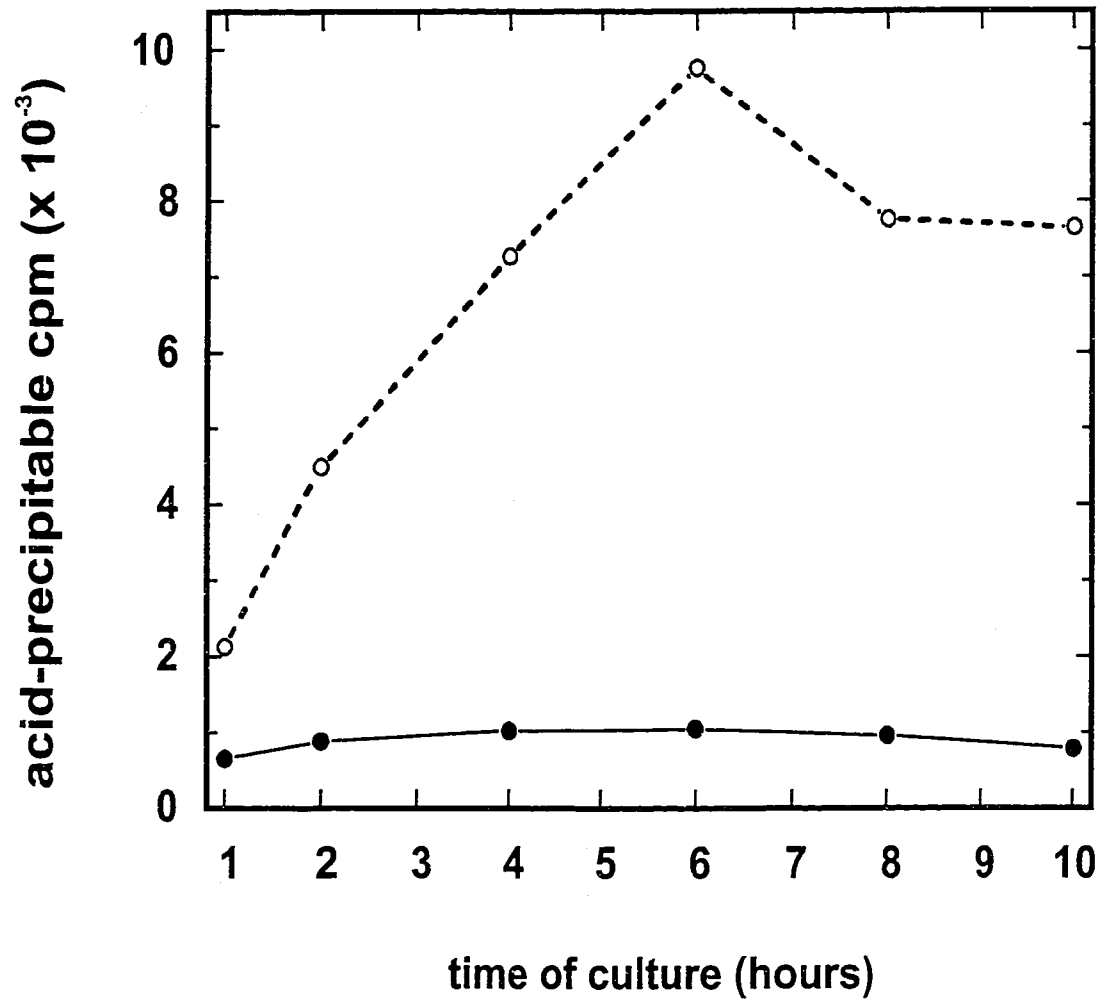
4.4 - Results

4.4.1 - Analysis of ³H-RNA from GM-CSF treated granulocytes

It had been demonstrated previously that stimulation of human neutrophils with ~50 ng/mL GM-CSF caused a 3-fold increase in [5-³H]uridine incorporation that peaked at 4 h, but dropped slowly thereafter [201]. I performed a similar time-course to confirm these results. Culturing of granulocytes with 20 ng/mL GM-CSF increased [6-³H]uridine incorporation ~10-fold by 6 h (Figure 4.1). This represents a sizable stimulation of transcription. Although radiolabeling did vary between donors, I found that the average increase with GM-CSF was slightly higher than the 3-fold published previously [201]. Promyelocytic HL-60 cells possess GM-CSF receptors [236]. Stimulation of HL-60 with

Figure 4.1. Time-course of [³H]uridine uptake into acid-precipitable RNA of cultured human granulocytes. Human granulocytes were cultured in 6-well tissue culture plates under standard culturing conditions either with (○) or without (●) 20 ng/mL GM-CSF (2×10^6 cells and 10 μ Ci [^{6-³H]uridine per well). At the indicated time (in h), cells were harvested by trituration, washed with PBS, and transferred to 1.5 mL screw-cap polypropylene microcentrifuge tubes. Cells were suspended in 500 μ L cold 0.15 M NaCl, 20 mM Tris/HCl (pH 7.5). Cold 5 N HCl was added (25 μ L per tube), and the suspension allowed to stand on ice for 20 min. The tubes were microcentrifuged, and the supernatant discarded. The pellet was washed once with 200 μ L cold 0.25 N HCl, and was dissolved in 150 μ L 0.1 N NaOH at room temperature. Dissolved pellets were counted in a liquid scintillation counter with 10 mL scintillation fluid. Each point shown in the graph is the average of 2 values obtained from one donor.}

-67-
Figure 4.1



— ● — without GM-CSF

-- ○ -- with GM-CSF

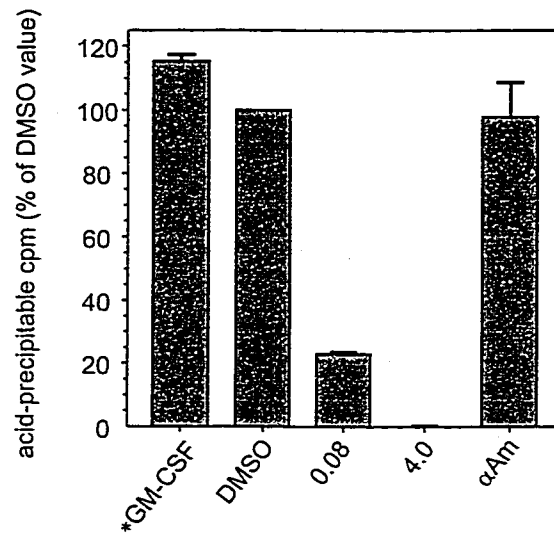
GM-CSF also caused an increase in radiolabeling of RNA (Figure 4.2A, compare DMSO and GM-CSF bars), but this increase was not as large as that observed with neutrophils (Figure 4.2B).

Next I determined the type of RNA that was being synthesized in granulocytes in response to GM-CSF (e.g., rRNA from RNA polymerase I, mRNA from RNA polymerase II, or tRNA from RNA polymerase III) by using inhibitors of transcription. These results are shown in Figure 4.2. High concentrations of actinomycin D (e.g., 4.0 $\mu\text{g/mL}$) inhibit transcription of all RNA species completely, while lower concentrations (0.01-0.08 $\mu\text{g/mL}$) inhibit rRNA synthesis more selectively [237]. At the concentration used in this experiment, α -amanitin inhibits RNA polymerase II activity specifically [187], but can be slow to manifest complete inhibition in intact cells [238,239]. To verify that the inhibitors were functioning as expected, their effect on [^3H]uridine-labeling of HL-60 cells was tested (Figure 4.2A). Compared to radiolabeling of vehicle-treated cells (i.e., DMSO bar), amanitin had no significant effect, 0.08 $\mu\text{g/mL}$ actinomycin D reduced incorporation by ~80%, and 4.0 $\mu\text{g/mL}$ actinomycin D completely eliminated radiolabeling. The majority of ^3H -RNA in HL-60 cells was ribosomal (18S, 28S and most likely 32S and 45S) as demonstrated by electrophoresis on a formaldehyde agarose gel (Figure 4.3, ^3H -RNA panel, HL-60 lane with black lettering). This corroborated the inhibitor data.

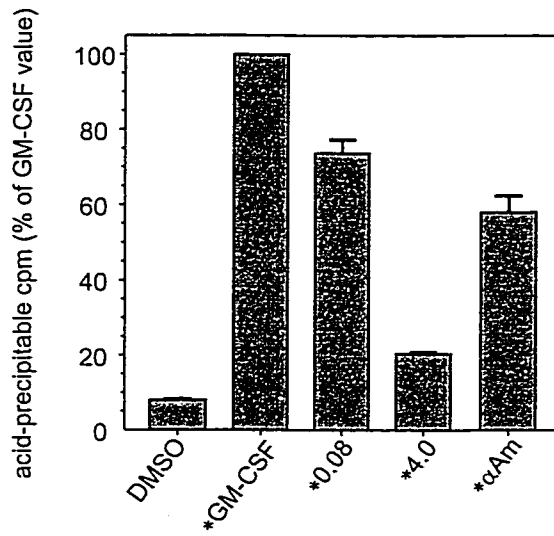
In contrast to the HL-60 data, [^3H]uridine-radiolabeling of GM-CSF stimulated granulocytes was inhibited more strongly by α -amanitin than by low concentrations of actinomycin D (Figure 4.2B, compare αam and 0.08 bars). This suggests that more radiolabeled RNA is of polymerase II than of polymerase I origin. When neutrophil

Figure 4.2. Effect of transcription inhibitors on ^3H -RNA synthesis in HL-60 cells (*part A*) and human granulocytes (*parts B and C*). Cells were cultured in 6-well tissue culture plates at 2×10^6 cells per well in 2 mL DMEM + 10% FCS (HL-60) or RPMI 1640 + 10% autologous serum (granulocytes). Wells contained either 0.08 $\mu\text{g/mL}$ actinomycin D (*0.08 bars*), 4 $\mu\text{g/mL}$ actinomycin D (*4.0 bars*) or 5 $\mu\text{g/mL}$ α -amanitin (*$\alpha\text{Am bars}$*). All wells also contained 0.1% DMSO as a solvent. Plates were incubated at 37°C in a humidified, 5% CO_2 atmosphere for 60 min. Ten μCi [5,6- ^3H]uridine (granulocytes) or 5 μCi [5- ^3H]uridine (HL-60) was added per well. GM-CSF (20 ng/mL) was also added where indicated by an *asterisk*. The incubation was continued for 4.5 h. Cells were harvested by trituration, washed with PBS, and transferred to 1.5 mL screw-cap polypropylene microcentrifuge tubes. Cells were suspended in 500 μL cold 0.15 M NaCl, 20 mM Tris/HCl (pH 7.5). Cold 5 N HCl was added (25 μL per tube), and the suspension allowed to stand on ice for 20 min. The tubes were microcentrifuged at high-speed for 4 min at 4°C , and the supernatant discarded. The pellet was washed once with 200 μL cold 0.25 N HCl, and dissolved in 250 μL 0.1 N NaOH at room temperature. Dissolved pellets were counted in a liquid scintillation counter with 10 mL scintillation fluid. Each bar is the mean of 3 values $\pm$ SEM. Bars are expressed as percentage of the DMSO value (*part A*, HL-60 cells) or percentage of the GM-CSF value (*parts B and C*, granulocytes). *Graph C* depicts the same data as *graph B* except that background cpm from the 4 $\mu\text{g/mL}$ actinomycin D sample has been subtracted from all bars marked with an *asterisk*.

A



B



C

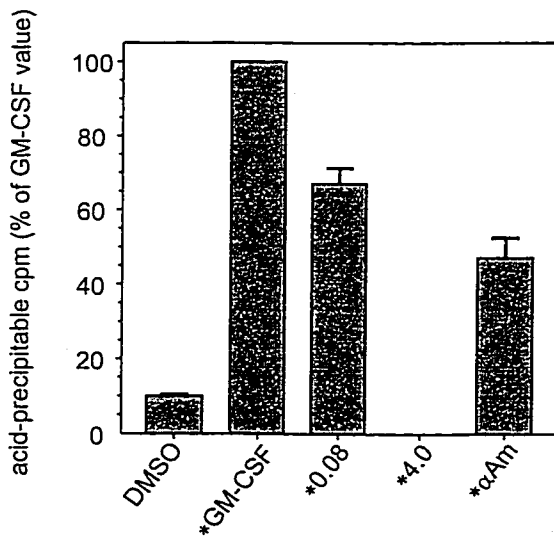
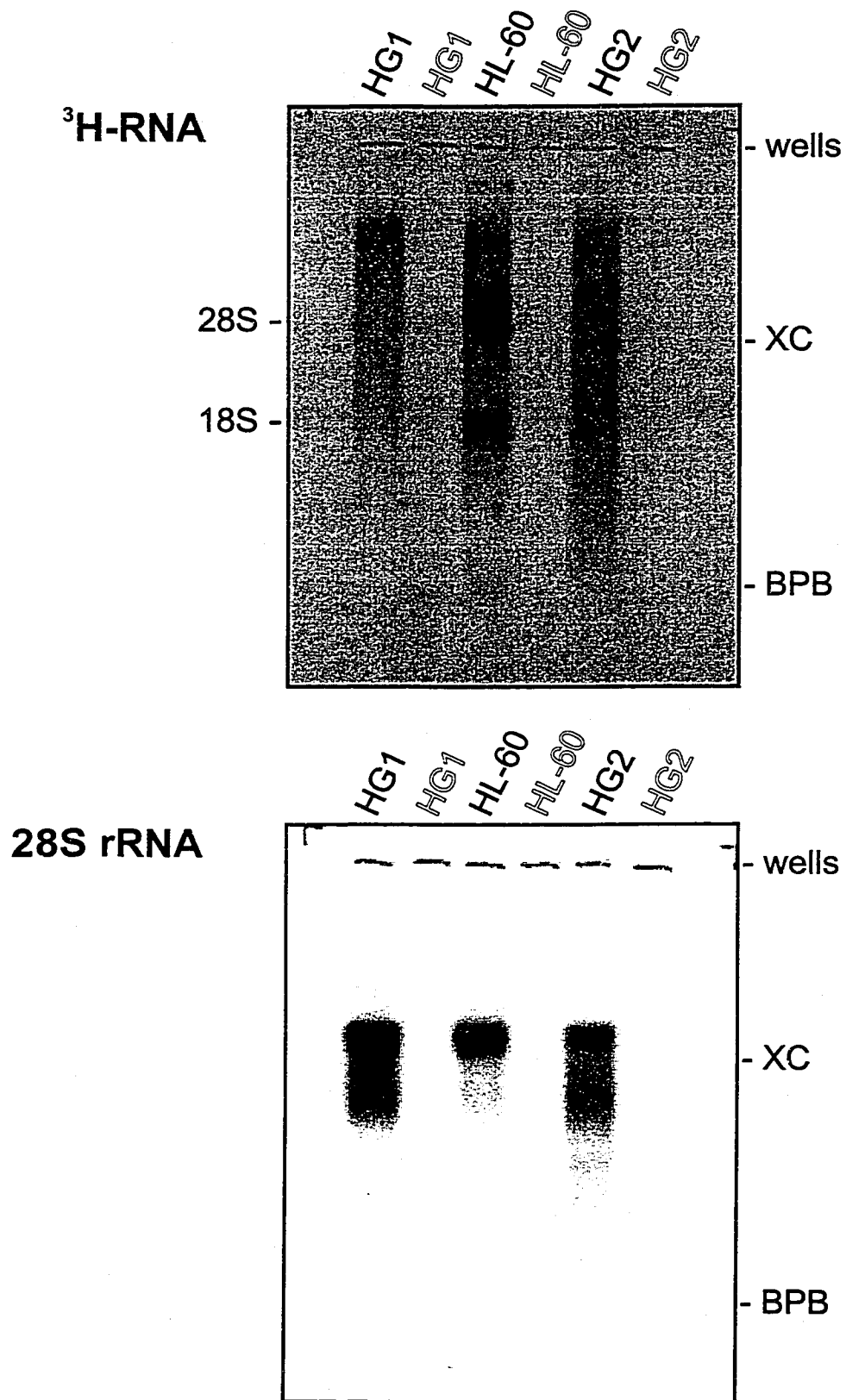


Figure 4.3. Analysis of ^3H -RNA from human granulocytes and HL-60 cells by agarose gel electrophoresis. 10^7 cells were radiolabeled with 50 μCi [5,6- ^3H]uridine for 6 h under standard culturing conditions. Cultures of granulocytes also contained 20 ng/mL GM-CSF. Total RNA was isolated from the radiolabeled cells as described in Chapter 2. Approximately 500 ng of RNA ($\sim 2 \times 10^6$ cell-equivalents of granulocytes and $\sim 5 \times 10^4$ cell-equivalents of HL-60) was electrophoresed on a formaldehyde-denaturing, 1.2% agarose gel, as described previously [177]. The gel was washed at room temperature for 20-30 min in water and then in $10\times\text{SSC}$. The gel was transferred to a nylon membrane under vacuum with $10\times\text{SSC}$ until ~ 40 mL had passed through the gel. The membrane was allowed to air-dry, and was fixed by baking in a desiccated oven at 80°C for 2 h. Residual tracking dye was removed from the membrane by washing at 50°C for 30-45 min in $2\times\text{SSC}$ / 0.1% SDS. The membrane was air-dried, sprayed with En 3 Hance Spray Surface Autoradiography Enhancer according to manufacturer's instructions, and exposed directly to x-ray film at -80°C for 48 h. The autoradiograph is shown in the *^3H -RNA panel*. Fluorography agent was then removed from the membrane by washing for 15-30 min at room temperature in methanol, followed by washing for 30-60 min at 50°C in $2\times\text{SSC}$ / 0.1% SDS. Northern blotting was performed on the membrane as described in Chapter 2, using a cDNA fragment representing nucleotides 1405-4933 of human 28S rRNA. The resulting autoradiograph is shown in the *28S rRNA panel*. HG1, human granulocyte donor #1; HG2, human granulocyte donor #2; XC, position of xylene cyanol; BPB, position of bromophenol blue. Lanes marked with black lettering contain intact RNA. Lanes marked with white lettering were digested with 100 $\mu\text{g/mL}$ DNase-free pancreatic RNase for 30 min at 37°C prior to electrophoresis.

-70-
Figure 4.3



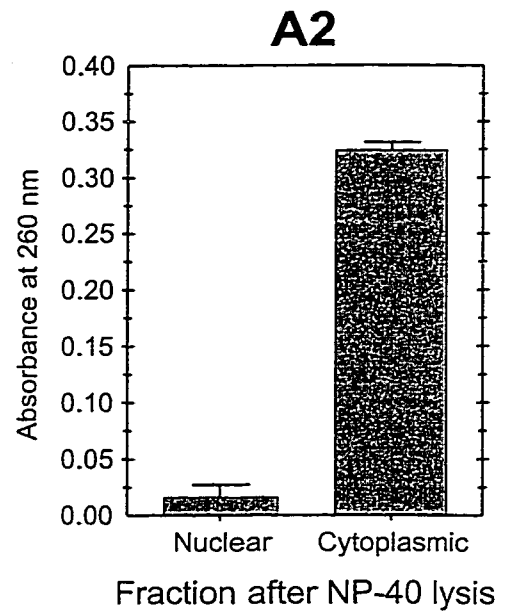
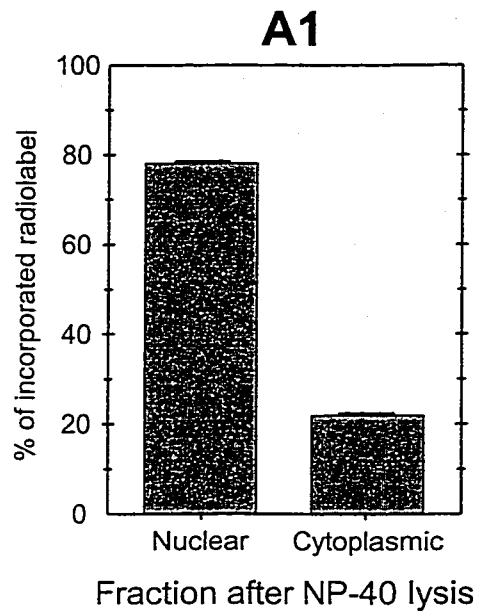
radiolabeled RNA was electrophoresed on an agarose gel, there was no evidence of specific ^3H -rRNA bands; only a heterogeneous smear could be detected (Figure 4.3, ^3H -RNA panel, HG1 and HG2 lanes with black lettering). Despite the absence of radiolabeled rRNA, 28S rRNA was detected by Northern blotting of the same membrane (Figure 4.3, 28S rRNA panel). The smears of granulocyte ^3H -RNA varied in size from >10 kb to <2 kb. Beaulieu *et al.* also found no evidence of radiolabeled rRNA from human neutrophils, but the smears that they detected were of smaller average size (<5 kb to <2 kb) [201]. The reason for this size discrepancy is not known. Since $4\text{ }\mu\text{g/mL}$ actinomycin D should halt all transcription, the 4.0 bar in Figure 4.2B likely represented [^3H]uridine that was not removed during washing (308 cpm or $<0.01\%$ of total input radiolabel). This background was subtracted from all GM-CSF-treated values to generate panel C of Figure 4.2, but this had no significant effect on the relative height of the bars nor the interpretation of the data. In Figure 4.3, digestion with DNase-free pancreatic RNase prior to electrophoresis completely eliminated the signals, verifying that all radiolabeled species were RNA (Figure 4.3, white-lettered lanes).

There was no indication of rRNA synthesis in GM-CSF-treated human granulocytes and at least 50% of the ^3H -RNA was of polymerase II origin based on inhibition by α -amanitin. The contribution of RNA polymerase III was then addressed by high-salt precipitation. High-salt (e.g., 1.5 M LiCl) will not precipitate mature tRNA due to its small size and high double-stranded character [240]. Of the ethanol-precipitable radiolabel from [5,6- ^3H]uridine-labeled, GM-CSF-stimulated granulocytes (6 h pulse), only $10.8 \pm 3.2\%$ ($\bar{x} \pm \text{SEM}$, $n = 6$) was not precipitated by LiCl. However, even this estimate is likely high

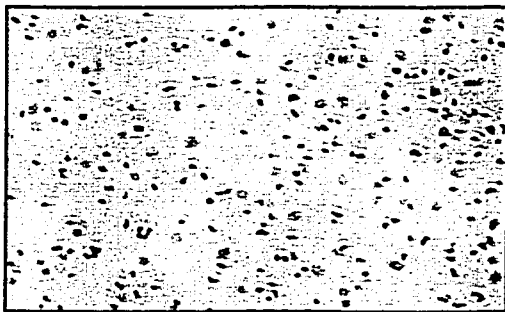
because unincorporated NTP, which can be precipitated by ethanol/sodium acetate, is not precipitated by high-salt [241]. Therefore, at least 90% of the ^3H -RNA was derived from RNA polymerase II transcription.

The end-product of almost all RNA polymerase II transcription is polyadenylated, cytoplasmic mRNA. Surprisingly, only $24.7 \pm 1.8\%$ ($\bar{x} \pm \text{SEM}$, $n = 4$) of the high-salt precipitable ^3H -RNA bound to oligo(dT) cellulose, indicating that most of the radiolabel was likely not polyadenylated. The cellular localization of the ^3H -RNA was investigated next. The majority was localized to the nucleus after a 6 h pulse, as demonstrated by 3 different criteria. (i) Separation of total RNA into nuclear and cytoplasmic fractions by NP-40 lysis revealed that ~80% of the radiolabel was in the nuclear fraction (Figure 4.4A1). This was not the result of inefficient extraction because >90% of rRNA was found in the cytoplasmic fraction as measured by A_{260} (Figure 4.4A2). (ii) Light microscopy of cellular autoradiograms revealed that the majority of silver grains were localized over the nucleus, and also that the synthesis of ^3H -RNA varied considerably from cell to cell (Figure 4.4B2 and B3). (iii) When intact cells are treated (in the absence of SDS) with hot phenol at pH 5.1, nuclear hnRNA is extracted inefficiently and tends to remain in the phenol phase or aqueous/phenol interphase [231]. $88 \pm 2\%$ ($\bar{x} \pm \text{SEM}$, $n = 3$) of the radiolabel from granulocytes remained in the phenol phase and interphase during such an extraction. The significance of the apparent nuclear retention of nonpolyadenylated RNA polymerase II transcripts will be discussed in sections 4.5.2 and 4.5.3.

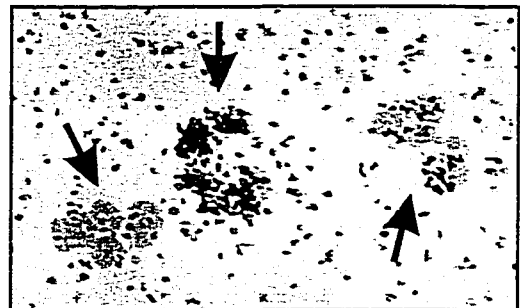
Figure 4.4. Radiolabeled RNA from GM-CSF-stimulated human granulocytes is concentrated in the nucleus. Part A1. Distribution of ^3H -RNA in human granulocytes. 10^7 granulocytes were cultured under standard conditions for 6 h in the presence of 20 ng/mL GM-CSF and 10 μCi [5,6- ^3H]uridine. Cells were harvested by trituration, washed with PBS, and nuclear and cytoplasmic RNA isolated as described in section 4.3.1. Bars are the means of 4 determinations $\pm$ SEM. Part A2. Distribution of RNA mass in human granulocytes. Nuclear and cytoplasmic RNA was isolated from 5×10^7 granulocytes as described in section 4.3.1. RNA mass was determined by its absorbance at 260 nm. Bars are the means of 3 determinations $\pm$ SEM. Parts B1-B3. Subcellular localization of ^3H -RNA in human neutrophils as determined by cellular autoradiography. Procedures are described in section 4.3.3. Representative light micrographs are shown (1000 $\times$ magnification) that depict both the radiolabeled neutrophils (*panels B2 and B3, arrows*) and the background signal due to the photographic emulsion alone (*panel B1*).



B1 (background)



B2



B3



4.4.2 - Differential Display RT-PCR of granulocyte total RNA

Despite the large percentage of [^3H]uridine incorporation into nuclear RNA, GM-CSF did induce significant changes in mature cytoplasmic mRNA. The technique of DDRT-PCR was used to identify transcripts that were upregulated in neutrophils stimulated with the cytokine. Using 14 combinations of anchored and arbitrary primers, eight fragments were cloned that appeared differentially expressed on the original polyacrylamide gel. These clones are denoted G26BS2.2 to G26CC2 in Table 4.II. Some fragments were not studied further because they matched uncharacterized ESTs. Also, a few fragments were amplified from what appeared to be a small amount of contaminating DNA, despite prior DNase I-digestion (e.g., clone G26CC2 in Table 4.II matched a cosmid DNA GenBank sequence). Clone A18AC5 and clone A9AC3 possessed sequence identity to mitochondrial 16S rRNA (96%) and the nucleolar RNA helicase Gu protein (89%) respectively [242] (Table 4.II). Since neutrophils contain few mitochondria and have no visible nucleoli on electron micrographs [1,38], it is surprising that these mRNAs were isolated. They were not analyzed further.

While attempting to isolate a full-length cDNA of one of the DDRT-PCR subclones from granulocyte RNA (data not shown), a clone that had apparently self-primed in the first-strand cDNA synthesis reaction was unexpectedly obtained. This clone was named A4AFL8 and its sequence matched 99% (over 316 bp sequenced) to the large transcript of hTegt (Table 4.II). Stimulation with GM-CSF produced no change in the level of this mRNA (Figure 4.5D). The study of hTegt protein will be presented in greater detail in Chapter 6.

The study of DDRT-PCR fragment G26B was pursued for two reasons. (i) It showed

Table 4.II
Summary of cloned DDRT-PCR fragments derived from granulocyte total RNA

Procedures are described in section 4.3.5. Examples of Northern blots are shown in Figure 4.5. Naming of clones was based on the DDRT-PCR procedure. For example, clone G26BS2.2 was *E. coli* clone "S2.2" that had been subcloned from DDRT-PCR fragment G26B. This fragment was amplified in a PCR reaction containing G-anchored primer and arbitrary primer #4.8 (Table 4.I) (primer #26 in Table 1 of Bauer *et al.* [234]). "B" refers to the position of the fragment from the top of the DDRT-PCR sequencing gel (i.e., second from the top).

Clone Name	Primers Used*	Insert Size (bp)**	GenBank Match***			Effect of GM-CSF on Northern Blot
			Name	Accession #	% Sequence Identity	
G26BS2.2	4.1	250	<i>hsgk</i> mRNA	AJ000512 Y10032	97%	10-fold increase
G26BS2.7	4.1	250	EST	T23061	100%	ND****
G26BS2.10	4.1	250	EST	AA086003	99%	no signal
G26BS3.3	4.1	250	<i>hsgly</i> intron 2	M90058	100%	no effect
A18AC5	4.3, 4.10	220	mitochondrial 16S rRNA	J01415	96%	ND
A9AC3	4.3, 4.6	250	Gu protein mRNA	U41387	89%	ND
G26DC5	4.1	200	EST	AA715639	67%	ND
G26CC2	4.1	200	cosmid DNA sequence	270049	73%	ND
A4AFL8	none	1500	large hTEGT mRNA	X75861	99% of 316 bp sequenced	no effect

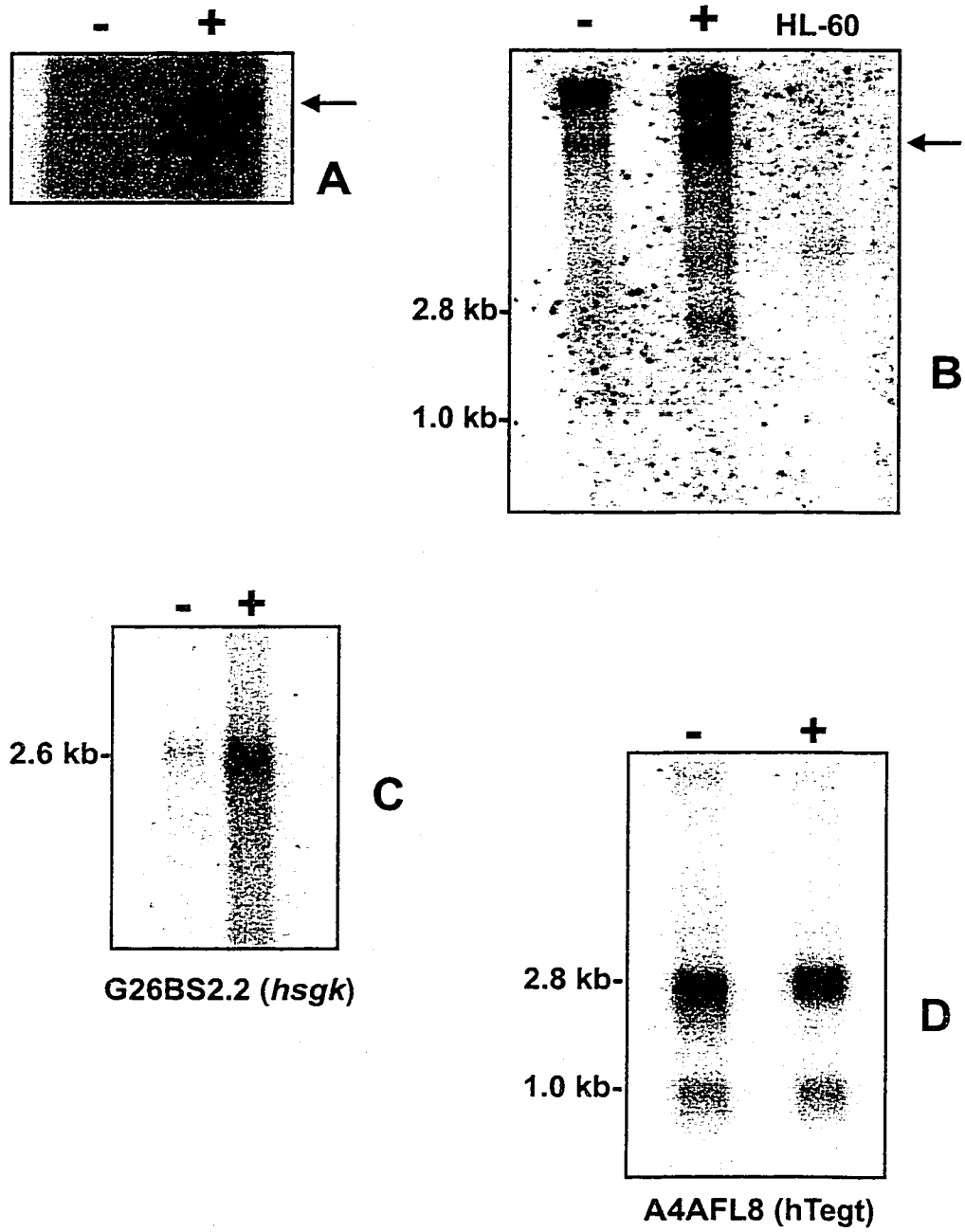
* see Table 4.I

** approximate size determined by agarose gel electrophoresis

*** Last BLAST search on January 21, 1998.

**** not determined

Figure 4.5. DDRT-PCR of human granulocyte total RNA. Differential display RT-PCR was performed on RNA isolated from granulocytes cultured with (+) or without (-) GM-CSF as described in section 4.3.5. One upregulated band(s) (denoted fragment G26B) was visualized by autoradiography of the DDRT-PCR sequencing gel, and is shown in *panel A*. *Panel B.* Northern blot of granulocyte and HL-60 RNA probed with G26B. Blotting was carried out on total RNA from 2×10^7 granulocytes (- and + lanes) and 2 μ g total RNA from HL-60 cells. The blot was probed with an [α - 32 P]dCTP-labeled G26B fragment (second PCR amplification product) that had been primed with primer #4.1 (see Table 4.I). *Panel C.* Northern blot of granulocyte RNA probed with subclone G26BS2.2. Blotting was carried out on total RNA from 3×10^7 granulocytes (- and + lanes). The blot was probed with the [α - 32 P]dCTP-labeled insert from subclone G26BS2.2 (see Table 4.II) that had been primed with primer #4.1 (see Table 4.I). The insert had been excised from the vector by digestion with *Eco*RI and *Bam*HI and purified on an agarose gel. *Panel D.* hTEGT mRNA expression in human granulocytes is not affected by GM-CSF stimulation. Northern blotting was performed on total RNA from 2×10^7 granulocytes (per lane), using the random-primed ~900 bp *Eco*RI / *Hind*III fragment of I.M.A.G.E. clone #207657 as a probe. All RNA isolation and Northern blotting procedures are detailed in Chapter 2.



the largest increase with GM-CSF on the original polyacrylamide gel (Figure 4.5A, arrow). and (ii) Multiple species were subcloned from this band (Table 4.II, clones G26BS2.2 to G26BS3.3). G26B was amplified with anchored primer 4.1 annealing to both forward and reverse strands. However, this was not surprising since internal amplification of AU-rich mRNA sequences with the anchored oligo(dT) primer alone has been noted as a common occurrence [243]. In panel A of Figure 4.5, there are three bands in the + GM-CSF lane. Despite selecting only the upper band, reamplified G26B was still a heterogeneous mixture because multiple bands were detected on a Northern blot when this fragment was used as a probe (Figure 4.5B). DDRT-PCR fragments that are composed of multiple species have been noted previously [244]. One band (indicated by an arrow in Figure 4.5B) was upregulated strongly with GM-CSF (compare - and + lanes). This transcript was of interest because of its large size (>5 kb). However, the DDRT-PCR probe that detected this band was never identified because, despite many attempts, it could not be subcloned. No signal was detected in total RNA from HL-60 cells with the exception of a faint band near the position of 28S rRNA (Figure 4.5B). Apparently, G26B was amplified from RNA species that were more abundant in mature granulocytes than in promyelocytic HL-60 cells.

Of the three G26B subclones tested on Northern blots, only one was affected by GM-CSF. Subclone G26BS2.2 matched human *sgk* mRNA in its 3'-UTR, and demonstrated a 10-fold increase with GM-CSF treatment (Table 4.II and Figure 4.5C). Human *sgk* will be described in more detail in section 4.4.3. Curiously, subclone G26BS3.3 matched the human serglycin gene within intron 2 (Table 4.II). I originally believed that this fragment was derived from contaminating DNA, but, as detailed in section 4.4.4, the intron signal could

be detected readily on Northern blots.

4.4.3 - Analysis of the upregulation of *hsgk* in GM-CSF-stimulated human granulocytes

The study of *hsgk* mRNA on granulocyte blots proved difficult with the G26BS2.2 fragment because it was small (~250 bp) and AT-rich. A partial *hsgk* cDNA was obtained from the I.M.A.G.E. consortium (clone #207120) [245], and its sequence verified by DNA sequencing. This I.M.A.G.E. fragment worked well as a probe and was used on all subsequent *hsgk* blots. The upregulation of the 2.6 kb *hsgk* was confirmed to be ~10-fold when granulocytes were stimulated with 20 ng/mL GM-CSF for 3 h (Figure 4.6A1 and A2, compare No and GM). When varying concentrations of GM-CSF were tested, 0.01 ng/mL did not induce *hsgk* expression, while 0.1 ng/mL did (Figure 4.6B1 and B2). A GM-CSF concentration of 0.1 ng/mL has been shown to delay the onset of apoptosis [69] and to induce cell polarization [71], but 0.01 ng/mL had no effect. Therefore, the dose-response of *hsgk* mRNA was consistent with a true receptor-mediated effect of GM-CSF and not with that of a minor contaminant. *Hsgk* mRNA levels increased between 0.01 and 1 ng/mL, but declined slightly at 10 ng/mL (Figure 4.6B2). The reason for this effect is not understood, but was seen in RNA from 2 different donors.

All Northern blots presented thus far used total RNA isolated from granulocytes stimulated with GM-CSF for 2-3 h. In the next experiment, a time-course of *hsgk* expression was performed. In Figure 4.7, the 2.6 kb *hsgk* mRNA was induced within 30 min with 1 ng/mL GM-CSF. This expression peaked at ~3 h, and then slowly decreased. At 12 h the *hsgk* mRNA was still elevated ~9-fold compared to granulocytes cultured in the absence of

Figure 4.6. **Expression of *hsgk* mRNA in human granulocytes and HL-60 cells.** Panel A1. Northern blot to detect *hsgk* mRNA. Blot was probed with the random-primed 1.2 kbp *EcoRI* / *NotI* fragment of I.M.A.G.E. clone # 207120 (*hsgk* blot), stripped and reprobed with a random-primed cDNA fragment representing nucleotides 1405-4933 of human 28S rRNA (28S blot). *No*, total RNA from 2.5×10^7 human granulocytes cultured for 3 h; *GM*, total RNA from 2.5×10^7 human granulocytes cultured for 3 h with 20 ng/mL GM-CSF; *Dexa*, total RNA from 2.5×10^7 human granulocytes cultured for 3 h with 1 μ M dexamethasone; *HL-60*, 5 μ g total RNA from HL-60 cells; *2d RA*, 5 μ g total RNA from HL-60 cells differentiated with all *trans*-RA for 2 d; *4 d RA*, 5 μ g total RNA from HL-60 cells differentiated with all *trans*-RA for 4 d. Panel A2. Quantitation of *hsgk* band intensity in panel A1. Signal intensities in panel A1 were quantified with PhosphorImager technology. Shown is a bar graph of *hsgk* signal intensity, normalized to the corresponding 28S loading control. Panel A3. Prolonged exposure of panel A1 (HL-60 lanes only). An arrow depicts the expected position of *hsgk* mRNA. Panel B1. Effect of GM-CSF concentration on *hsgk* mRNA levels in human granulocytes. 10^7 granulocytes were cultured for 3 h with the indicated concentration of GM-CSF, and total RNA was isolated from the cells. Northern blotting was carried out for *hsgk* and 28S rRNA as described for panel A1 above. Panel B2. Quantitation of *hsgk* band intensity in panel B1. Signal intensities in panel B1 were quantified with PhosphorImager technology. Shown is the bar graph of normalized *hsgk* signal versus GM-CSF concentration.

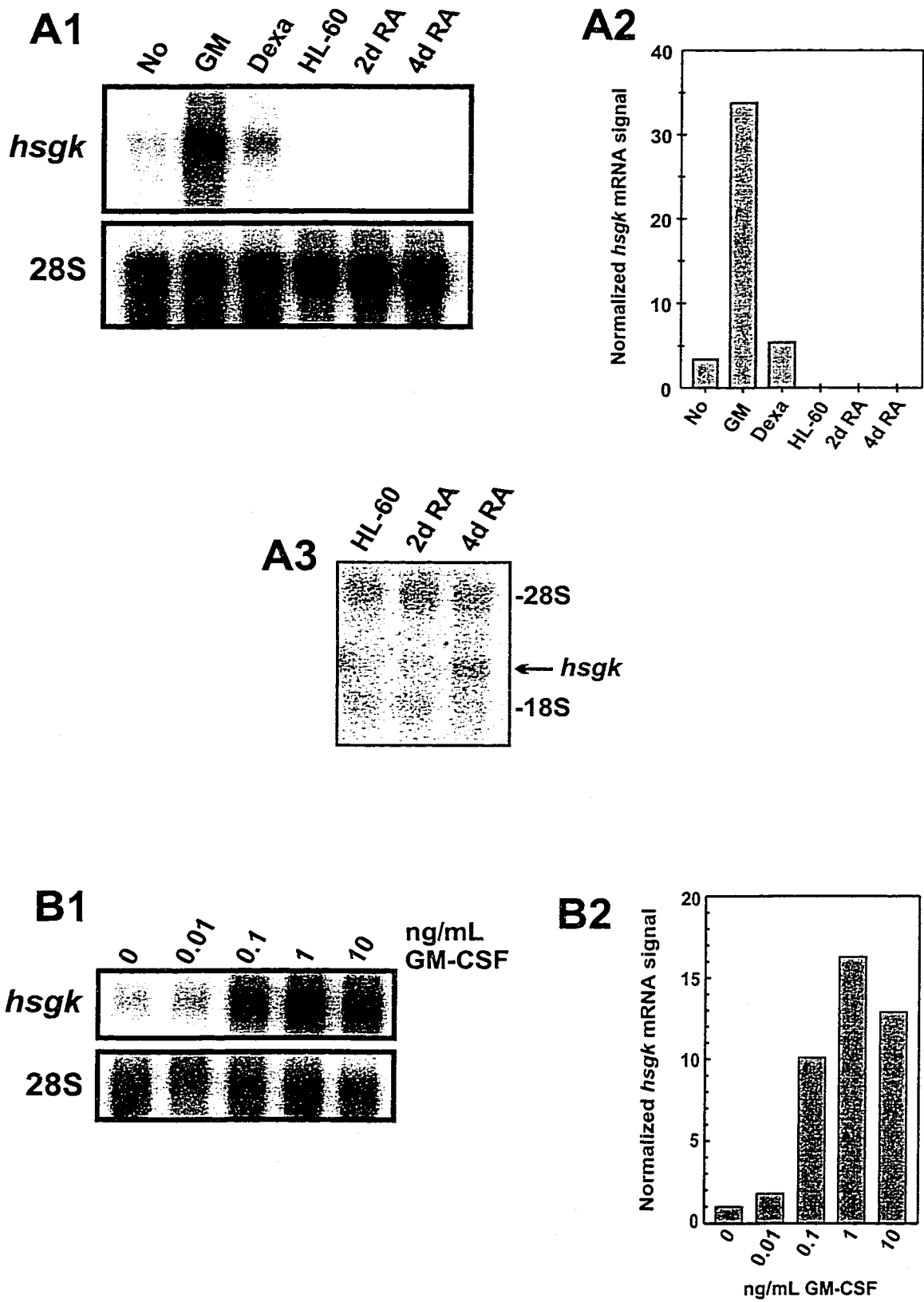
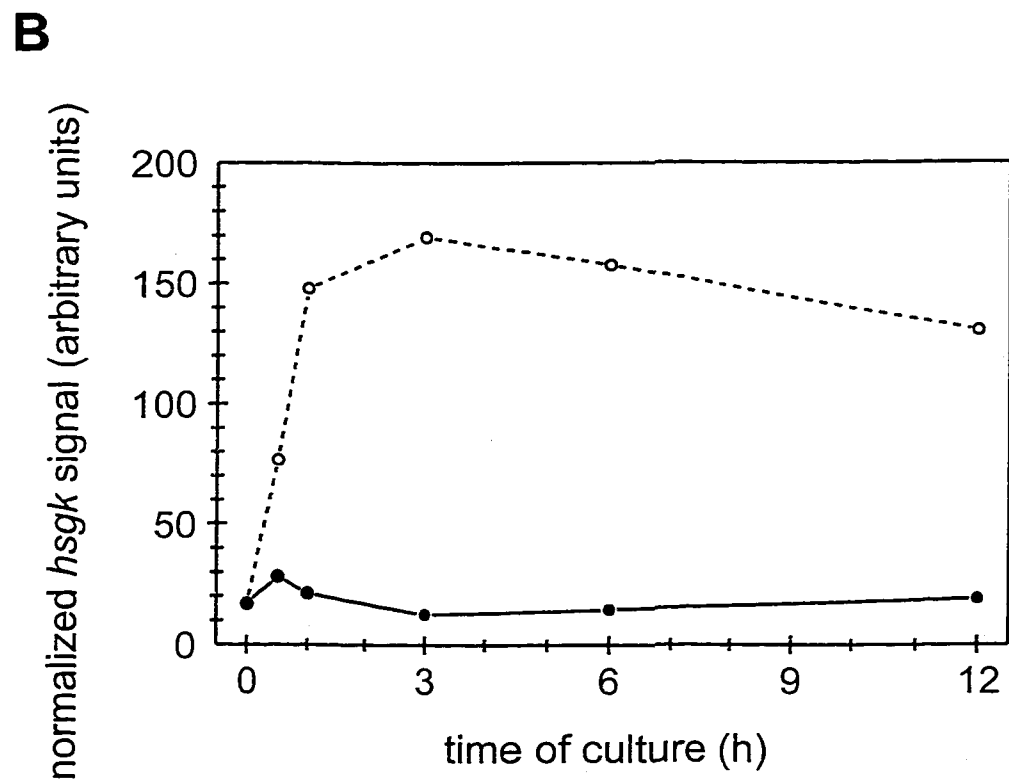
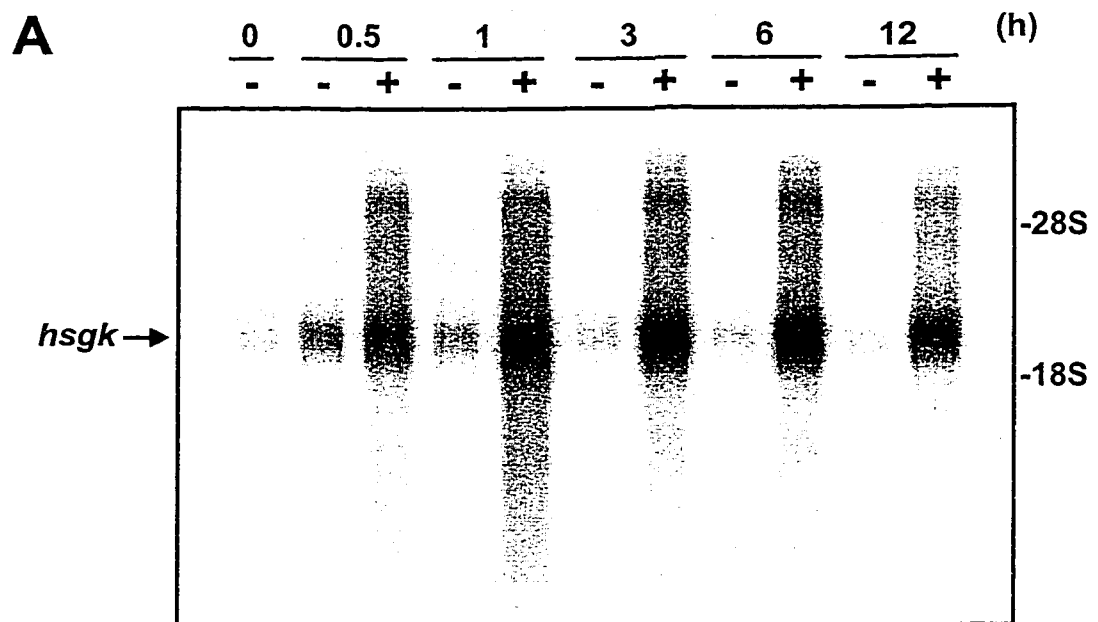


Figure 4.7. Time-course of *hsgk* mRNA expression in human granulocytes - effect of GM-CSF-stimulation. All procedures are outlined in Chapter 2. Part A. Northern blot for *hsgk*. Human granulocytes were cultured with (+) or without (-) 1 ng/mL GM-CSF, and total RNA was isolated from the cells after the indicated amount of time (h). Total RNA equivalent to 1.5×10^7 cells ($\sim 3 \mu\text{g}$) was electrophoresed per lane of a formaldehyde agarose gel and was transferred by vacuum to a nylon membrane. The membrane was probed with the random-primed 1.2 kbp *EcoRI* / *NotI* fragment of I.M.A.G.E. clone # 207120. Shown is the Northern blot, visualized by PhosphorImager screen. The positions of 28S rRNA, 18S rRNA and *hsgk* are highlighted. Part B. Quantitation of Northern blot shown in part A. The intensity of the *hsgk* signal was quantified by PhosphorImager technology, and the signals normalized with a human 28S rRNA probe. Shown is a plot of the normalized signal intensity versus time of cell culture. ● *solid line*, cultured without GM-CSF; ○ *dashed line*, cultured with 1 ng/mL GM-CSF.



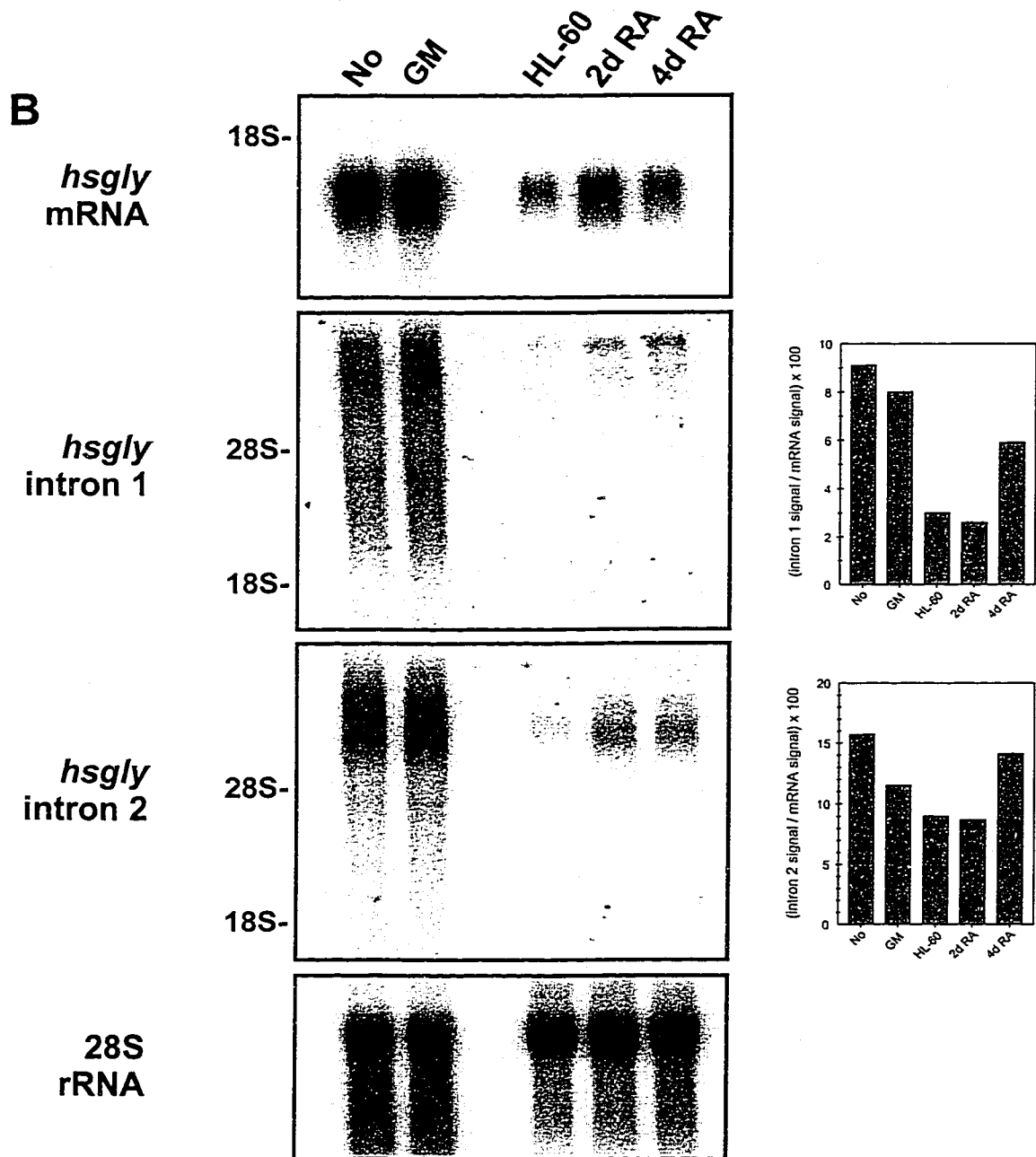
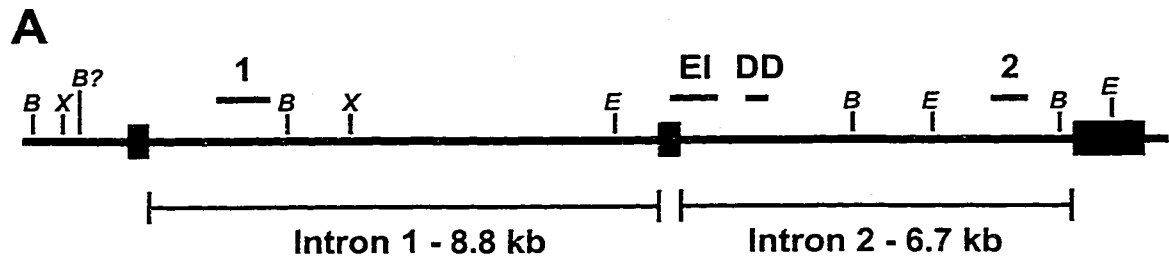
GM-CSF. Despite its rapid induction, increased *hsgk* expression persisted for a substantial amount of time, considering the short life-span of the neutrophil. Interestingly, *hsgk* mRNA levels were elevated slightly in unstimulated cells at 30 and 60 min (Figure 4.7A, - lanes and Figure 4.7B, ●). It is possible that the cell isolation and/or *in vitro* culturing may have triggered this response.

In contrast to the original study performed in rat mammary tumour cells [215] but consistent with recent studies in human hepatoma cells [216], 1 μ M dexamethasone did not increase levels of *hsgk* mRNA in human granulocytes (Figure 4.6A1 and A2, Dexa). In initial experiments with other myeloid cell-types, HL-60 cells and RA-differentiated HL-60 cells showed no detectable *hsgk* mRNA (Figure 4.6A1 and A2, HL-60, 2d RA and 4d RA). However, if the blot was exposed for longer periods, a faint *hsgk* signal was visible, especially in HL-60 cells differentiated with RA for 4d (Figure 4.6A3).

4.4.4 - Serglycin hnRNA in human granulocytes and HL-60 cells

Because an intronic region of *hsgly* had been amplified from granulocyte total RNA (see Table 4.II, clone G26BS3.3), Northern blotting was performed to verify that (i) the signal arose from RNA and not from contaminating DNA, and (ii) the signal showed an increase in GM-CSF-treated granulocytes. The complete sequence of the *hsgly* gene has been reported previously and is illustrated in part A of Figure 4.8 (GenBank accession #M90058) [224]. In Figure 4.8A, the region of *hsgly* intron 2 that was amplified in DDRT-PCR and subcloned as G26BS3.3 is labeled DD. For unknown reasons, plasmid DNA could not be isolated from this subclone after the first mini-prep. Therefore, other probes were

Figure 4.8. Northern blotting of human granulocyte and HL-60 total RNA with serglycin (*hsgly*) cDNA and intron probes. Part A. Diagrammatic representation of the human serglycin gene (16.7 kbp). Exons (3 in total) are denoted as black boxes. Introns (2 in total) and flanking DNA sequences are denoted as thick black lines. *B*, *Bgl*I restriction site; *B?*, possible *Bgl*I restriction site; *X*, *Xho*I restriction site; *E*, *Eco*RI restriction site. *DD*, sequence corresponding to DDRT-PCR clone G26BS3.1; *I*, *hsgly* intron 1 probe described in part B; *2*, *hsgly* intron 2 probe described in part B and intron 2 region amplified in Figure 4.10; *EI*, exon 2 / intron 2 region amplified in Figure 4.10. Part B. Northern blots of *hsgly* species. *No*, total RNA from 3×10^7 human granulocytes cultured for 3 h; *GM*, total RNA from 3×10^7 human granulocytes cultured for 3 h with 20 ng/mL GM-CSF; *HL-60*, 6 μ g total RNA from HL-60 cells; *2d RA*, 6 μ g total RNA from HL-60 cells differentiated with all *trans*-RA for 2 d; *4 d RA*, 6 μ g total RNA from HL-60 cells differentiated with all *trans*-RA for 4 d. Shown is one membrane (representative of 2 experiments) that had been probed, stripped and reprobed with the following random-primed, [α - 32 P]dCTP-labeled fragments. *hsgly* mature mRNA, 1.1 kbp RT-PCR fragment from ~40 ng HL-60 poly(A)⁺ RNA using primer #4.11 and 4.12 (30 cycles of 96°C/30 s, 58°C/20 s, 72°C/2 min); *hsgly* intron 1, 867 bp PCR fragment from 1 ng *Pst*I/*Bam*HI-digested HL-60 DNA using primer #4.15 and 4.16 (36 cycles of 95°C/30 s, 51°C/20 s, 72°C/1 min); *hsgly* intron 2, 595 bp PCR fragment from 1 ng *Pst*I/*Bam*HI-digested HL-60 DNA using primer #4.13 and 4.14 (36 cycles of 95°C/30 s, 56°C/20 s, 72°C/1 min); *28S rRNA*, cDNA fragment representing nucleotides 1405-4933 of human 28S rRNA. PCR primers are listed in Table 4.I. All fragments were purified on agarose gels prior to random-priming. Northern blots were quantified by PhosphorImager technology; serglycin intron 1 and 2 levels (relative to mature mRNA levels) are plotted to the right of their respective blots.



generated by PCR. Two PCR fragments were amplified from HL-60 DNA representing a region of *hsgly* intron 1 and intron 2 (labeled 1 and 2 respectively in Figure 4.8A). In addition, a *hsgly* cDNA fragment was amplified by RT-PCR of HL-60 poly(A)⁺ RNA. These PCR fragments were then used to probe Northern blots of granulocyte and HL-60 total RNA.

Human serglycin mRNA of ~1.3 kb was strongly expressed in both granulocytes and HL-60 cells (Figure 4.8B, mRNA, lanes No and HL-60). Stimulation of granulocytes with GM-CSF for 3 h had little effect on the level of mRNA (Figure 4.8B, *hsgly* mRNA, compare lanes No and GM). Differentiation of HL-60 cells to granulocytic cells with RA also had little effect on the level of *hsgly* mRNA (Figure 4.8B, *hsgly* mRNA, lanes HL-60, 2d RA and 4d RA). When the same membrane was stripped and reprobed with the *hsgly* intron 1 fragment, a smear was detected in granulocyte total RNA ranging from ~2 kb to >>5 kb (Figure 4.8B, *hsgly* intron 1, lanes No and GM). Only a faint intron 1 signal was detected in HL-60 total RNA, which consisted primarily of a high molecular weight band of probably the full-length hnRNA; but some very faint smearing was also visible, especially with RA-differentiated HL-60 cells (Figure 4.8B, *hsgly* intron 1, lanes HL-60, 2d RA and 4d RA). Reprobing with the *hsgly* intron 2 fragment detected a smear at >6 kb with all RNA samples, although the signal intensity was higher with granulocyte RNA (Figure 4.8B, *hsgly* intron 2). When the intron signals were quantified by PhosphorImager, normalized to the mRNA signal, and plotted, some observations were made with both intron probes. (See the bar graph to the right of the corresponding blot in Figure 4.8.) (i) The signals decreased slightly when granulocytes were stimulated with GM-CSF. (ii) The granulocyte signal was higher than the HL-60 signal, although this was more prominent with the intron 1 probe. (iii)

Differentiation of HL-60 cells with RA showed an increase with both intron probes. Equal loading of RNA was verified in all lanes by probing for 28S rRNA (Figure 4.8B, 28S rRNA).

The question of whether the signals in Figure 4.8 arose from RNA or contaminating DNA was addressed. A similar blot was hybridized with an *Alu* repeat probe (data not shown). This *Alu* probe detected a smear in both granulocyte and HL-60 total RNA because ~5% of mRNA contain *Alu* repeats in their UTRs [246]. Treatment of RNA samples with DNase-free pancreatic RNase prior to electrophoresis completely abrogated this *Alu* signal. Considering that *Alu* repeats occur every 3-5 kbp in the human genome [247], this is strong evidence that contaminating DNA was not responsible for the Northern blot signals seen in Figure 4.8.

Synthesis of each intron probe by PCR generated only 1 fragment of the anticipated size, but the identity of each probe was not verified by sequencing. Southern blots were performed on HL-60 DNA with both the *hsgly* intron 1 and intron 2 probes to further verify their identity (Figure 4.9). The expected bands and those obtained experimentally are summarized in Table 4.III.

Figure 4.9. Southern blotting of HL-60 DNA with *hsgly* intron probes. HL-60 genomic DNA was digested with the indicated restriction enzyme (2 units/ μ g DNA) for 12 h at 37°C. An additional 2 units of enzyme were added per μ g of DNA, and the incubation continued for 6 h. Twelve μ g of digested DNA was electrophoresed per lane of a 0.8% agarose gel at 22 V for 16.5 h (electrophoresis buffer was 1 mM CDTA, 20 mM sodium acetate, 40 mM Tris/acetic acid (pH 7.8)). The gel was stained for 30 min in 0.1 μ g/mL ethidium bromide, and photographed under UV transillumination to visualize molecular weight standards (indicated in kbp to the left of each blot). The gel was rinsed in water, gently shaken in 0.25 N HCl at room temperature for 30 min, rinsed in water, gently shaken in 0.4 N NaOH at room temperature for 20 min, and rinsed in water. The gel was transferred to positively-charged nylon membrane (Hybond-N⁺) by upward capillary transfer in 0.4 N NaOH for 5 h. The membrane was washed in 2 $\times$ SSC, and was allowed to air-dry. Membranes were hybridized, washed, and exposed to PhosphorImager screens as described in section 2.5 of Chapter 2, except that hybridization was performed at 65°C without formamide. Probes were the same human serglycin intron probes used for Northern blotting in Figure 4.8.

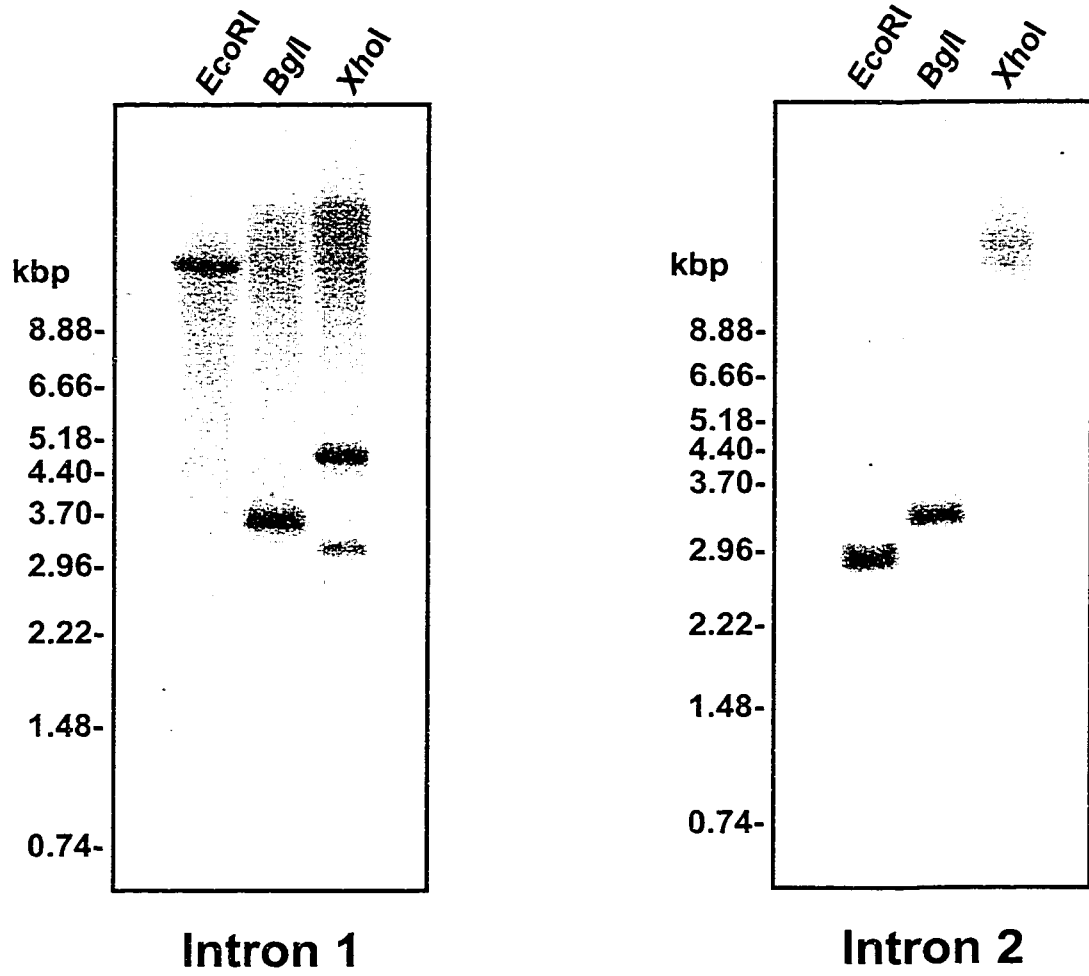


Table 4.III

Band sizes (predicted and obtained) on Southern blots probed with *hsgly* intron sequences.

Expected band sizes were calculated from GenBank accession #M90058 using DNASTar Windows MapDraw Version 3.03h software. Band sizes were obtained experimentally from Figure 4.9.

Restriction Endonuclease Used	Southern Blot Band Sizes (kbp)			
	INTRON 1		INTRON 2	
	Expected	Obtained	Expected	Obtained
<i>EcoRI</i>	>9.9	>8.9	2.924	2.9
<i>BglII</i>	4.465 (3.631*)	3.6	3.465	3.5
<i>XhoI</i>	4.725	4.7 (strong) 3.1 (faint)	>13	>>8.9

* See Figure 4.9 and text explanation.

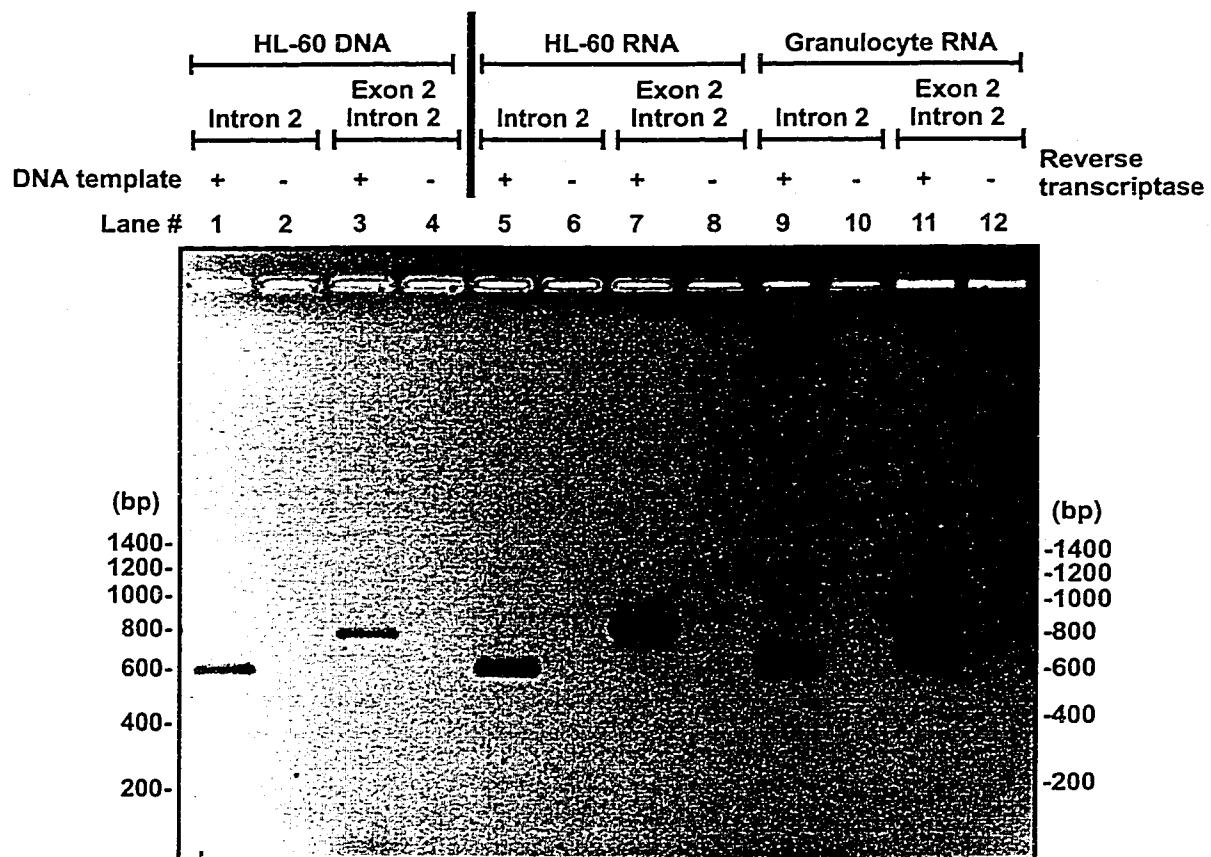
Although the intron 1 probe showed some background smearing on the Southern blot, both probes detected specific bands of the expected size. Based on the published *hsgly* sequence, the intron 1 probe should have detected a *BglII* fragment of ~4.5 kbp. However, further scrutiny of *hsgly* revealed the sequence ₇₇₇GnCCAGCCTGGC₇₆₆, which would be identical to the *BglII* restriction site GCCN₄↓NGGC if the n were a sequencing error [224]. Assuming that this *BglII* site was correct, the expected and experimentally obtained bands would both be 3.6 kbp (see Table 4.III). The intron 1 probe also detected an additional band at 3.1 kbp with *XhoI*-digested DNA, although the predominant band was of the expected size (Figure

4.9). This could not have been a cross-hybridization to another DNA sequence since only one band was detected in both *EcoRI* and *BglII* lanes. A likely explanation for this observation is a polymorphism in the HL-60 population with respect to a *XhoI* site. I conclude that the sizes of the *hsgly* intron probes and their banding patterns on Southern blots confirm their identity.

Serglycin intron sequences were detected in total RNA from both HL-60 cells and mature granulocytes. It is likely that these sequences represent unspliced introns in hnRNA. However, the possibility of persistent spliced introns cannot be ruled out using Northern blots exclusively. RT-PCR was employed to address this question. Two primer pairs were selected, one that amplified within *hsgly* intron 2 (amplified region marked as 2 in Figure 4.8A) and one that amplified across the exon 2 / intron 2 border (amplified region marked as EI in Figure 4.8A). Using HL-60 DNA as template, the intron 2 primers amplified the expected fragment of 595 bp and the exon / intron primers amplified the expected fragment of 760 bp (Figure 4.10, lanes 1 and 3). The amplification of bands was dependent upon the presence of input DNA (Figure 4.10, lanes 2 and 4). When RT-PCR was performed on HL-60 (differentiated with RA for 2 d) or granulocyte total RNA, both intron 2 and exon / intron amplimers of the expected size were obtained (Figure 4.10, lanes 5,7,9,11). These amplimers were derived from RNA and not contaminating DNA because no band was detected if reverse transcriptase was omitted from the reactions (Figure 4.10, lanes 6,8,10,12). Therefore, these results demonstrate that the intron hybridizations observed in Figure 4.8 were derived from *hsgly* hnRNA and not spliced out *hsgly* introns.

Figure 4.10. **Human granulocytes and RA-differentiated HL-60 cells possess detectable levels of *hsgly* hnRNA.** PCR reactions were performed with primer #4.13 and 4.14 (intron 2) or #4.17 and 4.18 (exon 2 / intron 2) using 36 cycles of 95°C/30 s, 56°C/20 s, and 72°C/75 s. All primers are listed in Table 4.I. PCR of HL-60 DNA (left side of gel). 0.1 ng of *Pst*I/*Bam*HI-digested HL-60 DNA was amplified per reaction (+ lanes), or omitted in the negative controls (- lanes). One-tenth of the 50 µL PCR reaction was loaded per lane of a 1.5% agarose gel. RT-PCR of HL-60 and granulocyte RNA (right side of gel). Total RNA from mature human granulocytes or HL-60 cells differentiated with RA for 2 d was isolated as described in Chapter 2. To eliminate small amounts of contaminating DNA, RNA samples were digested for 30 min at 37°C with RNase-free DNase I (~1 unit/µg RNA) containing placental RNase inhibitor (~1 unit/µg RNA). Samples were then deproteinized by phenol/ CHCl_3 extraction and concentrated by ethanol precipitation. ~200 ng (HL-60) or ~50 ng (granulocyte) total RNA was amplified per reaction. The only component omitted from the negative control reactions was the reverse transcriptase (- lanes). One-twentieth of the 50 µL PCR reaction was loaded per lane of a 1.5% agarose gel. The gel was electrophoresed at 80-100 V until the bromophenol blue tracking dye had migrated ~ $\frac{2}{3}$ down the length of the gel. The gel was stained for 20-30 min in 1 µg/mL ethidium bromide, destained for 5 min in water, and photographed under UV transillumination. The image has been inverted to improve contrast. Molecular weight markers (in bp) are shown at the sides of the gel. The expected product sizes are 595 bp (intron 2) and 760 bp (exon 2 / intron 2).

-88-
Figure 4.10



4.5 - Discussion

4.5.1 - Serum- and glucocorticoid-regulated kinase (*hsgk*)

Using DDRT-PCR, *hsgk* was identified in human granulocytes and found to be upregulated in response to GM-CSF. *Hsgk* mRNA levels increased rapidly with GM-CSF stimulation (within 30 min) and remained elevated for at least 12 h. This contrasted sharply with the time-course of *c-fos* induction by GM-CSF, where levels rose rapidly but were reduced to baseline values within 1-3 h (see Figure 3.4). The time-course of upregulation of *hsgk* in granulocytes in response to GM-CSF was similar to that previously observed in HepG2 human hepatoma cells in response to hypertonic media [216]. There is no indication from published reports as to how GM-CSF upregulates *hsgk* expression. The only transcription factors known to bind the rat *sgk* promoter are the glucocorticoid receptor, wild-type p53, Sp1, and Sp3 [215,248-250]. Human granulocytes do not upregulate *hsgk* in response to dexamethasone (see Figure 4.6A1), nor do they express detectable levels of p53 protein [16]. Basal transactivation of the rat *sgk* promoter was enhanced by PKA-mediated phosphorylation [250]. GM-CSF does increase tyrosine and serine/threonine phosphorylation in human neutrophils, but cAMP levels do not rise [164,165,201]; phosphorylation by other kinases could be involved. A better understanding will be gained once the human *sgk* promoter region has been sequenced. *Hsgk* mRNA has a short half-life of 20-30 min and contains several AU-rich regions in its 3'-UTR [216,217]. Enhancement of mRNA stability by GM-CSF-treatment may also contribute to increased *hsgk* levels, similar to that previously reported for IL-1 β mRNA [251].

From the data presented in Figure 4.6, one might conclude that an HL-60 cell

contains fewer *hsgk* mRNA molecules than a mature granulocyte. However, based on my observations, mature granulocytes contain 1/40 as much rRNA as HL-60 cells. Therefore, an equal mass of total RNA represents ~40x more granulocytes than HL-60 cells. Since the HL-60 signal in Figure 4.6 was too low to quantify reliably, the relative level of *hsgk* mRNA in each cell-type cannot be accurately determined. However, it is clear that *hsgk* mRNA represents a much higher proportion of the total RNA in GM-CSF-stimulated granulocytes than in HL-60 cells.

The function of *sgk* and identification of substrates that it may phosphorylate have yet to be elucidated. It has been suggested that *sgk* may be involved in G0 to S transition in response to mitogenic signals such as serum stimulation [217]. This would be of little consequence to human neutrophils because they are terminally differentiated and do not divide. Of greater interest is the finding that *hsgk* levels increase under hypertonic stress, suggesting that *hsgk* may play a role in protecting a cell from ion imbalances and reductions in cell volume [216]. One effect of GM-CSF on human neutrophils is to delay the onset of spontaneous apoptosis, this delay requiring both RNA and protein synthesis [70]. Intracellular acidification has been correlated with the onset of apoptosis in neutrophils and one of the hallmarks of apoptosis is cytoplasmic shrinkage [79]. GM-CSF stimulation of human neutrophils induces a rapid activation of Na^+/H^+ antiporters in the plasma membrane, leading to a cytoplasmic alkalinization of 0.1-0.25 pH unit [153,252]. This effect is transient and will temporarily protect against intracellular acidification. Induction of *hsgk*, which persists for at least 12 h, may provide a second wave of protection against ion imbalances, possibly by direct phosphorylation and activation of the Na^+/H^+ antiporter.

In future experiments, it would be useful to verify that *hsgk* protein levels are increased with GM-CSF. This is especially important in neutrophils, where translational capacity is low [1,38,171,185]. An antibody is available from the laboratory of Gary L. Firestone [215]. It would also be of interest to examine the effect of other proinflammatory mediators such as LPS, fMLP or G-CSF on the expression of *hsgk*. This would indicate whether *hsgk* induction is a general neutrophil response to stimulation or one that is specific to GM-CSF. To explore the function of *hsgk* in granulocytes, antisense oligonucleotides could be used to determine if *hsgk* mRNA levels are necessary for GM-CSF apoptotic delay. Despite many technical problems associated with manipulating peripheral blood neutrophils using molecular biology techniques, antisense oligonucleotide experiments have been reported [16,150,253]. *In vitro* translated rat *sgk* demonstrated no kinase activity as measured by autophosphorylation or by the substrates histone H1 and myelin basic protein [215]. Assuming that *sgk* is a kinase, it either requires factors that are not present in a standard kinase assay or it has a limited subset of protein substrates. Determining the substrates of this putative kinase is critical to understanding its function. Establishment of a stable *hsgk*-inducible cell-line would be a very valuable tool to identify proteins that may be phosphorylated in response to *hsgk* upregulation. This would not prove that *hsgk* has protein kinase activity, but it would demonstrate that expression of *hsgk* invokes protein phosphorylation, either directly or indirectly.

4.5.2 - Radiolabeling of RNA

Granulocytes show a significant increase in incorporation of [³H]uridine into RNA

when cultured with the cytokine, GM-CSF. Using selective inhibitors, electrophoretic separation and high-salt precipitation, at least 90% of this ^3H -RNA was found to originate from polymerase II transcription. The remaining radiolabeled RNA appeared to be of polymerase III origin. The lack of detectable polymerase I transcription was expected since mature granulocytes contain few ribosomes, no visible nucleoli, and low levels of 18S and 28S rRNA [1,38]. The few ribosomes that remain in a mature neutrophil were likely synthesized at an earlier stage of differentiation. Some of the 28S rRNA from these 'old' ribosomes did show partial degradation in both donors from Figure 4.3. The presence of few ribosomes does explain the low translational capacity of neutrophils (see Figure 3.1, protein graph). The lack of rRNA synthesis may also explain the low rates of [^3H]uridine incorporation into granulocytes (see Figure 3.1, RNA graph), since rRNA represents ~77% of the total RNA mass in a typical cell [254].

Despite the fact that the majority of ^3H -RNA was derived from polymerase II transcription, only ~25% of the radiolabel bound to oligo(dT). A similar percentage of ^3H -RNA (20-25%) extracted into the cytoplasmic fraction after NP-40 lysis. The question that remains unanswered is whether these two observations are related. Although most mature polymerase II transcripts have poly(A) tails (with the exception of e.g., histones [255]), binding of polyA⁺ mRNA to oligo(dT) cellulose may not be 100% efficient. Furthermore, cellulose can nonspecifically bind RNA [256,257]. Nevertheless, it is tempting to speculate that the 20% of ^3H -RNA is found in the cytoplasm and is polyadenylated. Conversely, 80% of ^3H -RNA may be found in the nucleus and not polyadenylated. If true, the presence of many nonpolyadenylated, polymerase II transcripts in the nucleus after a 6 h pulse of

[³H]uridine would be puzzling. Granelli-Piperno *et al.* also observed by cell autoradiography that most of the ³H-RNA was in the nucleus of 4 h-pulsed neutrophils [171].

The percentage of radiolabeled mRNA expected to be in the cytoplasm after a 6 h pulse with [³H]uridine can be estimated from previously published data. The calculations were based on four reports, each using a different experimental method (see Appendix II). Depending on the method, the calculated percent ³H-mRNA expected in the cytoplasm ranged from 13-64% (Appendix II). It is not known which of the estimates, if any, is correct. The proportion of nuclear and cytoplasmic polymerase II transcripts is difficult to determine accurately in cell-types where rRNA predominates. For example, values AII.1 and AII.2 in Appendix II were obtained by subcellular fractionation of HeLa and mouse L cells respectively. The 3-fold difference in % cytoplasmic ³H-mRNA between the 2 values can be attributed either to differences between cell-types or to techniques used to fractionate the cells. Given the range of % cytoplasmic ³H-mRNA determined by others (13-64%) and my observations for neutrophils (12-22%), I can only conclude that mature neutrophils may manifest a slow accumulation of cytoplasmic mRNA. The large range in the estimates of Appendix II makes it difficult to be confident. Analysis of specific transcripts would be a better approach. Analysis of *hsgly* introns and exons by Northern blotting of granulocyte total RNA is one such example and is discussed in section 4.5.3 below.

4.5.3 - Serglycin transcription

Before proceeding to discuss the analysis of *hsgly* hnRNA, the discrepancy in the literature regarding the expression of *hsgly* mRNA during HL-60 differentiation will be

addressed. My finding that the levels of *hsgly* mRNA change little in HL-60 cells after 2 and 4 d of all *trans*-RA differentiation is in agreement with that of Stellrecht *et al.* [230]. However, it is not consistent with the findings of Finstad *et al.*, who observed that *hsgly* mRNA levels decreased ~10-fold after 2 d of 1 μ M RA treatment [229]. The former results more closely reflect the *in vivo* scenario since *hsgly* mRNA levels drop only ~2-fold when promyelocytes differentiate to segmented neutrophils in bone marrow [227]. The reason for the discrepancy between different workers is not known. The promoter of the human serglycin gene contains a retinoid X receptor (RXR) response element [258]. If all-*trans*-RA is converted to the 9-*cis* form, it can activate the RXR and downregulate *hsgly* transcription [229]. Many cells have the capacity to degrade RA; confluent 10T1/2 cells can metabolize 1 μ M all-*trans*-RA, depleting it completely from the culture media by 48 h [259]. *Hsgly* mRNA would downregulate upon addition of RA, but would return to initial levels once the retinoid had been degraded. Once committed, a differentiated cell would be less affected by the loss of RA since differentiation is irreversible.

Probes specific to regions of *hsgly* introns 1 and 2 were shown to hybridize to total RNA extracted from both HL-60 cells and human granulocytes. Granulocyte intron signals were consistently higher than those of HL-60 cells. However, the signal intensities may be misleading because granulocytes contained less RNA (see section 4.5.1, second paragraph for further explanation). To circumvent this problem, intron signals were normalized by dividing by the mRNA signal; normalized values are plotted beside the corresponding Northern blots in Figure 4.8. While this was the best method available for standardizing, it does assume that the stability of *hsgly* mRNA is similar between promyelocytes and mature

neutrophils. This is a reasonable assumption, but still requires testing. Normalized *hsgly* intron signals dropped slightly when granulocytes were stimulated with GM-CSF but they were consistently higher than in HL-60 cells. I conclude that granulocytes are apparently slower to synthesize mature mRNA, so that a greater proportion of hnRNA is detected. Stimulation with GM-CSF likely reduced normalized intron signals by increasing hnRNA turnover.

Intron sequences could persist in three different types of hnRNA: (i) incompletely elongated transcripts, (ii) incompletely processed transcripts (e.g., partial splicing), or (iii) partially degraded hnRNA due to turnover of full-length transcripts [260-262]. Each intron probe detected a different range of granulocyte RNA sizes (see Figure 4.8), indicating that randomly degraded full-length hnRNA contributed little to the Northern signals. If splicing were the rate-limiting step, discrete bands of partially spliced intermediates would have been prevalent. Also, an intron proximal to the polyadenylated exon (i.e., intron 2) must wait for polyadenylation to occur before it can be spliced, but a distal intron (i.e., intron 1) can be spliced before transcription has completed [263]. If hnRNA processing (i.e., splicing or polyadenylation) were slow in mature granulocytes, one would expect that the normalized intron 2 signal would be equal to or greater than that of intron 1. The results are best explained by postulating that transcriptional elongation is the rate-limiting step. Higher amounts of *hsgly* intron 1 would be expected. Compared to HL-60 cells, mature human granulocytes expressed more normalized intron 1 signal ($\sim 3\times$) than normalized intron 2 signal ($\sim 1.5\times$). According to this model, mature granulocytes elongate *hsgly* hnRNA more slowly compared to promyelocytic HL-60 cells. Two additional observations help to support

this conclusion. (i) The *hsgly* intron 1 probe detects ≥ 2 kb of that transcript; the smallest size of RNA detected by the intron 1 probe in Figure 4.8 is ~ 2 kb. (ii) Smears were detected readily above and below the 2.6 kb *hsgk* mRNA band in Figure 4.7.

Granulocytes appear to be slow at synthesizing mature cytoplasmic mRNA. In addition to the smearing, discrete bands (likely representing partially spliced intermediates) can be seen in the intron 1 and 2 Northern blots of Figure 4.8 and the Northern blot of Figure 4.7 (with longer exposure). However, the evidence obtained from *hsgly* blotting suggests that the rate-limiting step was transcriptional elongation. This is also consistent with the observation that $\sim 75\%$ of ^3H -RNA would not bind oligo(dT) cellulose, since polyadenylation occurs after completion of elongation. Regulation of elongation is well established in prokaryotic and viral systems, but has only recently gained acceptance in eukaryotic systems [264]. Pausing or blocking of transcriptional elongation can occur at specific DNA sequences that are present in human genes such as *c-myc* (near the exon 1/intron 1 boundary) [265,266], *L-myc* (near intron 1) [267], and adenosine deaminase (near the 5' end of the gene) [268]. These pausing sequences may occur frequently throughout genes, so that the state of the transcriptional complex itself may be more important than the presence of specific DNA sequences [264]. The rate of transcriptional elongation by polymerase II can be modulated by ancillary factors. TFIIS (elongation factor SII), TFIIF, elongin (SIII), ELL, and ELL2 are examples of mammalian transcription factors that increase the rate of polymerase II transcription by suppressing pausing at sites within a gene [269-275]. All are general elongation factors; to my knowledge, no factor specific to a subset of genes has been identified, although the presence of pausing sequences within a particular gene will influence

elongation rate. Given their advanced state of differentiation, it is possible that mature granulocytes no longer possess one or several of these elongation factors, which would produce a general slowing of transcriptional elongation. Also of interest is the finding that neutrophils lack DNA-dependent protein kinase [87], a kinase whose phosphorylation of RNA polymerase II in its carboxyl terminal domain has been implicated in enhancing processivity of transcription [264,276].

Torelli *et al.* found by R_{ot} analysis that the sequence complexity of total poly(A)+ RNA dropped from 64,000 to 26,000 different sequences when HL-60 promyelocytes were differentiated to granulocytic cells with RA [200]. A general reduction in the synthesis of RNA is expected to lower the level of cytoplasmic mRNA in a cell. Slowed transcriptional elongation may have arisen during differentiation of granulocytes and may have produced the unusual phenotype of these cells (e.g., few subcellular structures such as ER and mitochondria, no rRNA synthesis, low translational capacity, etc.) by reducing mRNA levels. However, it is also possible that the granulocyte phenotype is responsible for slowing mRNA synthesis. Muralidhar and Johnson found that mature mRNA synthesis was reduced ~3-fold in mouse 3T6 fibroblasts under conditions of reduced protein synthesis [262]. In granulocytes, limiting amounts of transcription factors or polymerase machinery resulting from low rates of translation may be causing the 'problem'. Whether slowed synthesis of mature mRNA was responsible for the neutrophil phenotype or vice versa is not currently known, but would be an interesting area of further study. Curiously, Muralidhar and Johnson concluded that reduced protein synthesis in 3T6 cells caused a reduction in the processing rate for hnRNA, although nuclear ribonucleoprotein distribution appeared normal [262].

Although granulocytes may also be manifesting a slowness to process hnRNA, transcriptional elongation appears to be affected to a greater extent. The reason for this difference could also be studied further.

The trends in the *hsgly* intron Northern blots (Figure 4.8) were reproducible with two independent donors, but these trends may be exclusive to a small subset of genes. To determine whether this is a more general effect, other genes will have to be studied. *Hsgly* was used because it is an abundantly expressed message (i.e., signals were easy to detect on Northern blots), its gene has been completely sequenced, and it has a simple structure of 3 exons and 2 large introns. However, generation of specific intron probes for *hsgly* was not straightforward because *Alu* repetitive elements occurred once every ~1 kb [224]. In retrospect, *hsgly* was not the best candidate to examine the 'problems' of mature mRNA synthesis in granulocytes because mRNA expression was high (see Figure 4.8). To acquire a better candidate, I wanted to identify the DDRT-PCR fragment that hybridized to the granulocyte RNA band indicated with an arrow in Figure 4.5B. This RNA species was very large (>10 kb) and was likely not a mature mRNA. However, as mentioned in section 4.4.5, the DDRT-PCR fragment could not be cloned in *E. coli*. Direct sequencing of G26B was unsuccessful because it was a heterogeneous mixture of fragments and an adequate primer was not available (primer #4.1 annealed to both strands). The method of Li *et al.* to purify heterogeneous DDRT-PCR fragments by affinity capture on Northern blots would perhaps be suitable to identify this fragment in the future [244]. Once a homogeneous fragment had been amplified, it could be rendered asymmetric with respect to primer #4.1 by digesting with an appropriate restriction endonuclease, agarose gel purified, and sequenced.

Chapter 5

Protein synthesis in human granulocytes stimulated with GM-CSF.

5.1 - Summary

This chapter will study the effect of GM-CSF on protein synthesis in human granulocytes. Because the cytokine is known to delay the onset of apoptosis in these cells, its effect on pro-apoptotic and anti-apoptotic Bcl-2 family members was assessed by Western blotting. Of the four family members tested (Bcl-2, Bcl-X, Mcl-1 and Bax), mature human granulocytes express detectable levels of Bax protein only, and stimulation with GM-CSF for up to 24 h had no effect on this pattern of expression. When looking at total protein synthesis, the uptake of [³⁵S]Met into protein of human granulocytes was increased ~1.5-fold with 3-6 h of GM-CSF stimulation. I found that an unknown 26-27 kDa protein (denoted p27gran) demonstrated the greatest increase in radiolabeling on SDS-PAGE, representing a 2-3-fold relative increase with 3-6 h of GM-CSF treatment. This nonmembrane protein could be digested by proteinase K and V8 protease but was resistant to digestion by endogenous neutrophil proteases. The upregulation of p27gran was not inhibited by actinomycin D, suggesting a post-transcriptional regulation, and was not inhibited by chloramphenicol, suggesting that it was not encoded on the mitochondrial genome. Translation of p27gran was inhibited by cycloheximide to a lesser extent than that of other neutrophil proteins such as β/γ -actin, a property believed to be caused by the targeting of p27gran to the ER and secretory pathways. Hsp27, p27Kip1, hTegt, Bcl-2, Bcl-X_L and Gst π were eliminated as candidates for p27gran by either Western blotting, peptide mapping, Triton X-114 phase separation or peptide sequencing. Although I was unsuccessful in identifying p27gran, experiments were performed that would help future investigations. Identification of p27gran would have been of considerable interest since it demonstrated the

largest increase in translation when granulocytes were stimulated with GM-CSF for up to 6 h.

5.2 - Introduction

The uptake of radiolabeled amino acids into neutrophil protein is significantly lower than in other cell-types, indicating that PMNs have a low translational capacity (e.g., see chapter 3, Figure 3.1). Electron micrographs of neutrophils have revealed very few ribosomes and scarce ER [1,25,38]. It is generally believed that most proteins destined for secretion were synthesized during differentiation and stored in granules. Despite these observations, it is now known that neutrophils do indeed constitutively translate certain proteins such as actin, MHC class I, CR1, CR3 α -chain, Fc receptor [204], gelatinase [277], CAP [278], TGF- β 1 [279] and IL-1Ra [159]. Various stimuli can also induce neutrophils to synthesize proteins. Examples include Hsp70 and 85 in response to incubation at 42°C [280], 5-lipoxygenase, FLAP, IL-3 receptor α , IL-8 and IL-1Ra in response to GM-CSF [155-159], MIP-1 α , IL-8 and IL-12 in response to LPS [206-208], IFN- α and the cytosolic B subunit of the v-ATPase in response to G-CSF [117,205], and IL-1Ra in response to TNF α [159]. Although other inflammatory cells such as monocytes produce a greater amount of cytokine per cell, the total amount synthesized and secreted by neutrophils can be considerable due to the large numbers of PMNs present at the site of an acute infection [172]. Therefore, proteins known to be translated in neutrophils can have a significant impact on the induction, progression and resolution of inflammation.

GM-CSF increases [35 S]Met uptake into neutrophil protein, although this increase is

not as large as [^3H]uridine-uptake into RNA (see Chapter 4, Figure 4.1). As demonstrated by Waksman *et al.*, GM-CSF-stimulated human neutrophils assimilate 1.5-fold more [^{35}S]Met during a 2 h incubation compared to unstimulated cells [278]. They identified 10 proteins which showed increased radiolabeling on SDS-PAGE, and concluded that some were CAP because of their properties on ion exchange chromatography. However, none of these upregulated proteins was positively identified.

As mentioned previously, neutrophils spontaneously undergo programmed cell death *in vivo* as well as *in vitro* [70,73,78,82]. Using immunoblotting, others have demonstrated that these cells express the pro-apoptotic Bax protein, but do not express detectable levels of the anti-apoptotic proteins Bcl-2, Bcl-X_L, or Mcl-1 [20]. Overexpression of pro-apoptotic Bcl-2 family members directs a cell towards programmed death [281], although there are some exceptions to this rule [282,283]. The relative proportion of Bcl-2 members found in neutrophils is consistent with their eventual apoptotic fate. However, whether cytokine-stimulation can alter the relative proportion of these apoptotic factors has not been fully addressed. As demonstrated with transgenic mice, Bcl-2 expression in mature neutrophils does increase the survival of these cells [284]. The question remains whether expression of anti-apoptotic Bcl-2 members has any biological significance in normal, nontransgenic animals.

Two problems are addressed in this chapter. (i) Since GM-CSF can increase Bcl-2 levels in AML cells [285] and can prolong the survival of neutrophils, the effect of GM-CSF on expression of Bcl-2 family members in mature granulocytes was analyzed by immunoblotting. (ii) I wanted to determine if GM-CSF-treatment caused a *relative* increase

in translation of any protein(s) on SDS-PAGE, compared to unstimulated granulocytes. When Waksman *et al.* [278] identified proteins with increased [^{35}S]Met radiolabeling in GM-CSF-treated neutrophils, they had loaded equal amounts of protein per gel lane. Since short (i.e., 2 h) pulses were used, these increases may have represented an increase in translation or simply a change in the intracellular specific activity of [^{35}S]Met. Essentially the same experiment was repeated but the same amount of *incorporated radiolabel* was loaded per gel lane. An attempt was then made to positively identify any protein(s) demonstrating a relative increase in radiolabeling.

5.3 - Materials and Methods

5.3.1 - 1-D SDS-PAGE of radiolabeled granulocyte proteins

Granulocytes were cultured in methionine-free RPMI 1640 + 10% autologous serum containing 5-30 $\mu\text{Ci/mL}$ L-[^{35}S]methionine. Cells were collected, washed extensively with PBS, and solubilized by heating directly in SDS sample buffer (2% SDS, 10% glycerol, 1% 2-mercaptoethanol, 0.0005% bromphenol blue, and 62.5 mM Tris/HCl (pH 6.8)). Alternatively, cells were extracted with nonionic detergent (as specified in the text), diluted with SDS sample buffer and boiled for 2-3 min. Extracts were electrophoresed on 12% discontinuous SDS-polyacrylamide gels (Bio-Rad Protean II, 16 $\times$ 20 $\times$ 0.1cm) until the tracking dye had exited the bottom [178]. Resolving gels were stained with Coomassie Brilliant Blue R250 to visualize molecular weight standards, and were then impregnated with En 3 Hance fluorography agent following manufacturer's instructions. Gels were dried on chromatography paper and exposed to x-ray film at -80°C.

For N-terminal protein sequencing, precautions were taken to minimize blocking of the amino-terminus (Dr. Makoto Yaguchi, NRC Institute for Biological Sciences, Ottawa, personal communication). The resolving gel was allowed to polymerize for 48 h before pouring the stacking gel. Cell extracts were not boiled in SDS sample buffer; rather, they were heated at 65°C for 2 min prior to loading. Sodium thioglycolate (1 mM) was included in the upper (cathode) buffer. After electrophoresis, the resolving gel was electrophoretically transferred to PVDF membrane in 10% (v/v) methanol, 10 mM Caps/NaOH (pH 11.0) at 50V for 60 min. Total protein and radiolabeled protein were then visualized by Coomassie Brilliant Blue R250 staining and direct autoradiography, respectively. The band of interest was cut from the membrane with a razor blade and was subjected to N-terminal peptide sequencing using an Applied Biosystems Procise Sequencer (performed by Dr. Yaguchi's group).

5.3.2 - Triton X-114 extractions

Triton X-114 phase partitioning was used to partition insoluble, aqueous and membrane proteins, using a procedure similar to that described by Bordier [286]. A pellet of radiolabeled granulocytes was suspended in 100 μ L 0.14 M NaCl, 1 mM CDTA, 1 mM PMSF, 2 μ g/mL aprotinin and 20 mM Mops/NaOH (pH 7.2). 20 μ L Triton X-114 (precondensed several times in saline to remove aqueous-soluble contaminants) was added, vortexed, and the mixture allowed to stand on ice for 15 min. The mixture was centrifuged at 10,000 $\times$ g for 10 min at 4°C and the pellet (insoluble protein) retained for further analysis. The supernatant was heated at 37°C for 2 min, and then microcentrifuged at 10,000 rpm for

1 min at room temperature. The aqueous phase (upper) and Triton X-114 phase (lower) were separated. All 3 fractions were extracted 2 additional times to improve partitioning.

5.3.3 - Purification of radiolabeled proteins on SDS-PAGE

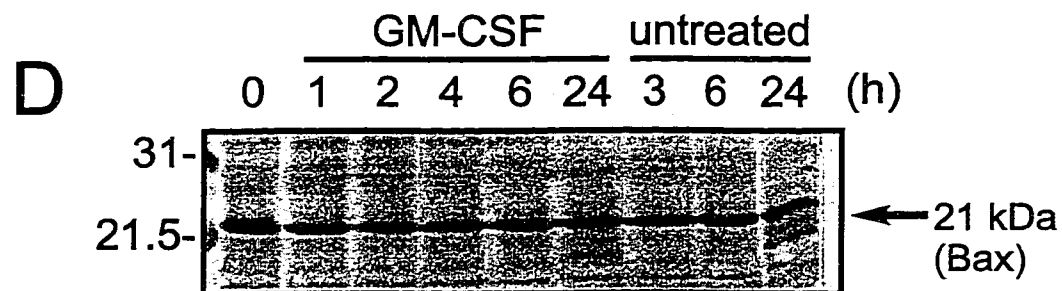
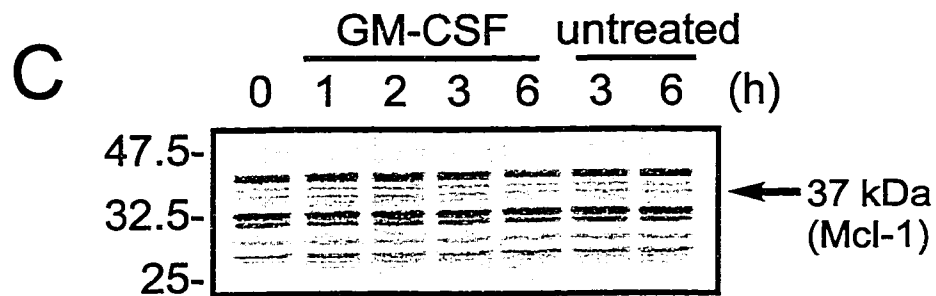
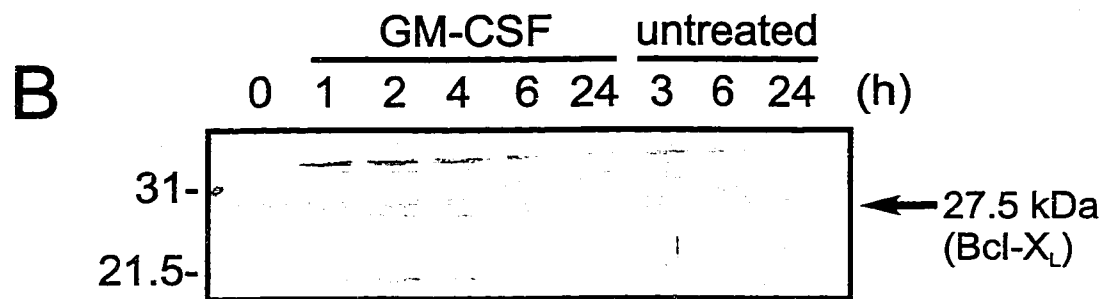
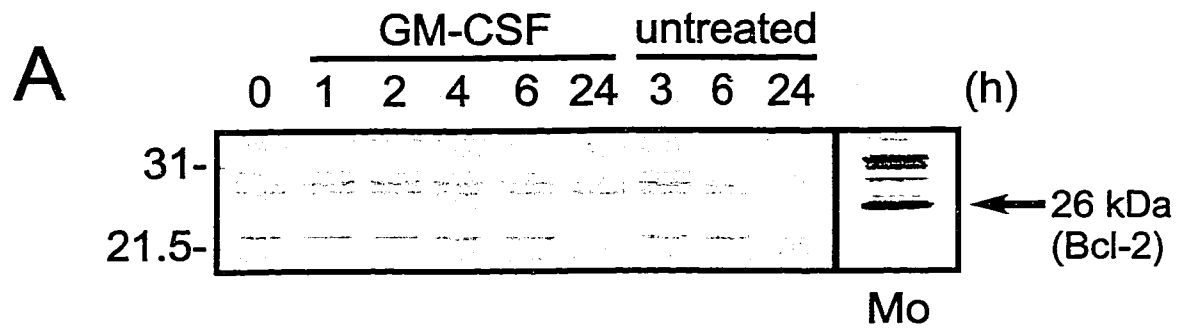
[³⁵S]Met-labeled proteins were electrophoresed on 12% discontinuous SDS-PAGE by the method of Laemmli [178]. Resolving gels were dried, unfixed and unstained, onto chromatography paper with a Bio-Rad gel dryer (60°C, 60 min, under vacuum), and were subsequently exposed to x-ray film at -80°C for a few days. Using the x-ray film as a guide, the bands of interest were cut from the dried gel with a razor blade. Gel pieces were rehydrated in 200 µL 0.5% SDS, pulverized, heated for 60 min at 65°C, then pulverized again. The SDS/gel mixture was centrifuged through a 0.22 µm Millipore Ultrafree-MC Durapore microcentrifuge filter. The filtrate containing the radiolabeled protein of interest was collected and used for further studies. Recovery of radiolabeled protein was typically 85-90%.

5.4 - Results

5.4.1 - Western blotting of Bcl-2 family members

To examine the effect of GM-CSF stimulation on the synthesis of Bcl-2 family members in human granulocytes, immunoblotting was performed using polyclonal antisera obtained from Dr. John C. Reed (Burnham Institute, La Jolla, California). As expected, unstimulated granulocytes possessed detectable levels of Bax protein, but little or no Bcl-2, Bcl-X_L or Mcl-1 (Figure 5.1, 0 and untreated lanes) [20]. Throughout 24 h of GM-CSF

Figure 5.1. Western blotting of human granulocyte extracts with antisera against Bcl-2 family members. Freshly isolated granulocytes (0 time point) were cultured for the indicated amount of time (in hours) with (GM-CSF lanes) or without (untreated lanes) 20 ng/mL GM-CSF. Extracts were prepared by boiling cells for 5 min directly in SDS/Mops buffer (2% SDS, 10% glycerol, 1% 2-mercaptoethanol, 0.0005% bromphenol blue, and 50 mM Mops/NaOH (pH 6.8)). 30 µg total protein was loaded per lane of a 12% SDS-PAGE gel and Western blotting carried out as described in Chapter 2, following additional instructions provided by Dr. John C. Reed. *Panel A.* Anti-Bcl-2. *Panel B.* Anti-Bcl-X_L. *Panel C.* Anti-Mcl-1. *Panel D.* Anti-Bax. Molecular weight standards are marked in kDa on the left side of each blot. The expected position of each Bcl-2 member is indicated by an *arrow* at the right of each blot. Mo, human peripheral blood mononuclear cell extract (20 µg total protein).



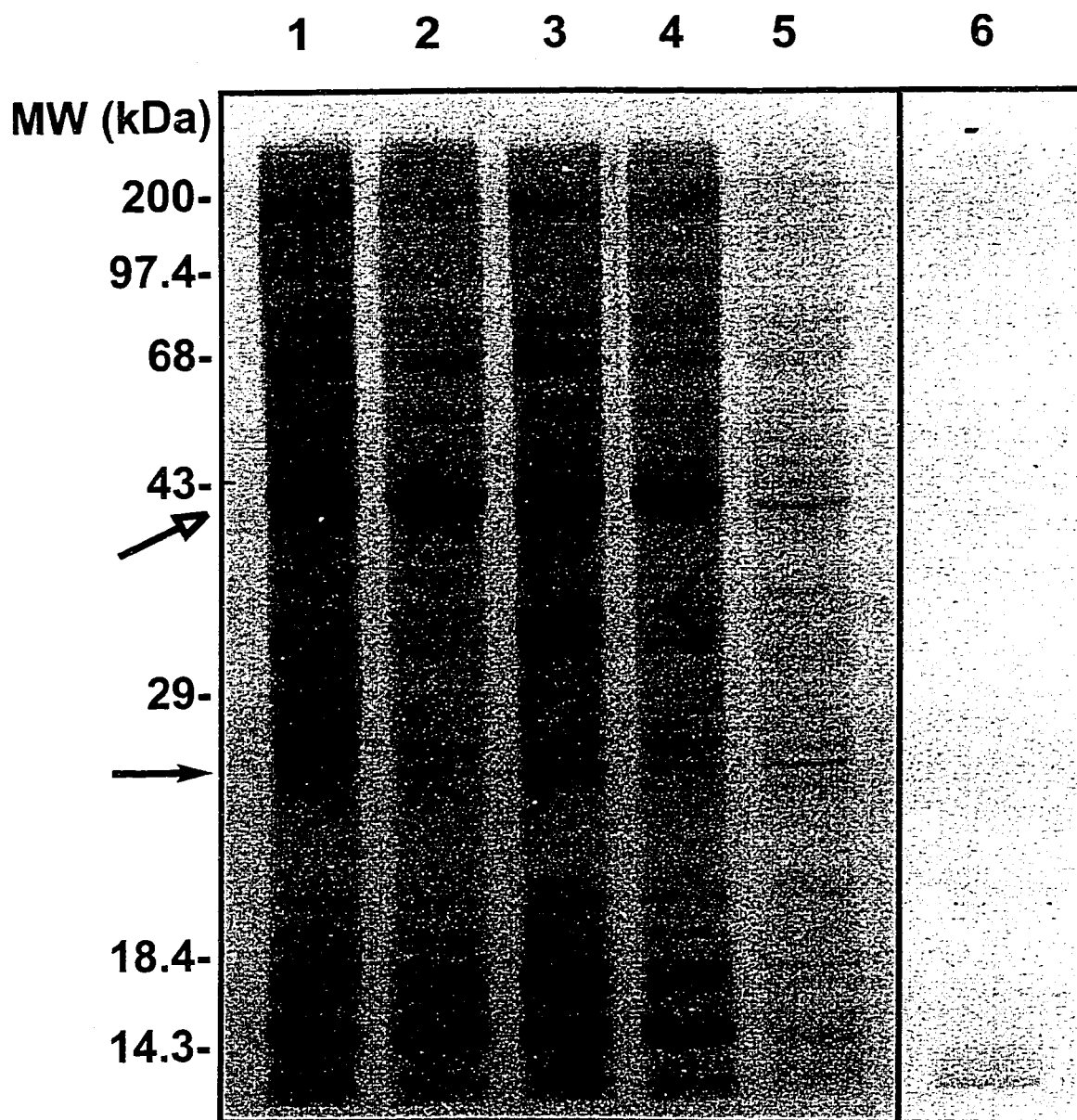
stimulation, this situation remained unchanged (Figure 5.1, GM-CSF lanes), although some degradation of Bax occurred by 24 h (Figure 5.1D). It is important to note that panels A,B and C of Figure 5.1 were overexposed to emphasize the lack of protein expression. Bcl-2 antisera was able to detect readily a band of ~26 kDa from human peripheral blood mononuclear cells (Figure 5.1A, lane Mo), demonstrating the activity of the antisera. Although no positive controls are shown for Bcl-X_L and Mcl-1, these same antisera have been used successfully by others [20,287,288]. Although only Bcl-X_L is highlighted in Figure 5.1B, its shorter pro-apoptotic form, Bcl-X_S (19 kDa), also was not detected with the antisera. Therefore, GM-CSF treatment does not alter the relative proportions of Bcl-2 family members in human granulocytes; Bax remains the predominant protein.

5.4.2 - [³⁵S]Methionine-radiolabeling of human granulocyte proteins

Culturing human granulocytes in the presence of 20 ng/mL GM-CSF resulted in a 1.2- to 1.5-fold increase in [³⁵S]Met uptake compared to unstimulated cells. The time of radiolabeling ranged from 3-6 h, but fold increases were similar at all time points. These data are similar to that obtained by Waksman *et al.*, who demonstrated a 1.5-fold increase over a 2 h incubation with ~6 ng/mL GM-CSF [278]. When extracts of granulocyte proteins were electrophoresed on 12% SDS-PAGE, many radiolabeled bands were observed (Figure 5.2). The open arrow in Figure 5.2 highlights β/γ-actin, a protein known to be abundantly synthesized in these cells [204].

When extracts from GM-CSF stimulated and unstimulated granulocytes were compared on SDS-PAGE, only one prominent band was reproducibly upregulated with GM-

Figure 5.2. Fluorography of [³⁵S]methionine-labeled human granulocyte proteins electrophoresed on 12% SDS-PAGE. Granulocytes were radiolabeled for 3 h with 7.5 μ Ci/mL L-[³⁵S]Met and extracts electrophoresed as described in section 5.3.1. Radioactive concentrations of extracts were quantified by liquid scintillation counting, and the same amount of radioactivity was loaded per gel lane. Prior to radiolabeling, granulocytes were preincubated for 1 h with: *Lane 1.* no addition, *Lane 2.* 2 μ g/mL actinomycin D, *Lane 3.* 20 ng/mL GM-CSF, *Lane 4.* 20 ng/mL GM-CSF and 2 μ g/mL actinomycin D, *Lane 5.* 20 ng/mL GM-CSF and 10 μ g/mL cycloheximide. Granulocyte cultures from lanes 1-4 also contained 0.04% DMSO as a solvent carrier. *Lane 6.* Same as lane 3 except that the cell extract was digested with 300 μ g/mL proteinase K for 2 h at 37°C prior to electrophoresis. An *open arrow* denotes the position of β/γ -actin. A *closed arrow* denotes the position of the upregulated p27gran.



CSF treatment (compare lanes 1 and 3 of Figure 5.2). This band (designated p27gran hereafter) had an apparent molecular weight of 26-27 kDa and is indicated by the closed arrow in Figure 5.2. All lanes of Figure 5.2 have been standardized to total incorporated radiolabel (i.e., the same number of cpm's have been loaded per lane). Therefore, p27gran has demonstrated the largest relative increase in translation of all protein visible on the 12% gel. In two independent trials, quantitation by PhosphorImager technology revealed that this band showed a 3.0- and 2.6-fold increase with GM-CSF stimulation compared to a 0.8- and 1.3-fold increase for β/γ -actin. Because it can be seen readily on SDS-PAGE autoradiography of total cell extracts, p27gran is very likely an abundant intracellular protein.

5.4.3 - Characteristics of radiolabeled p27gran

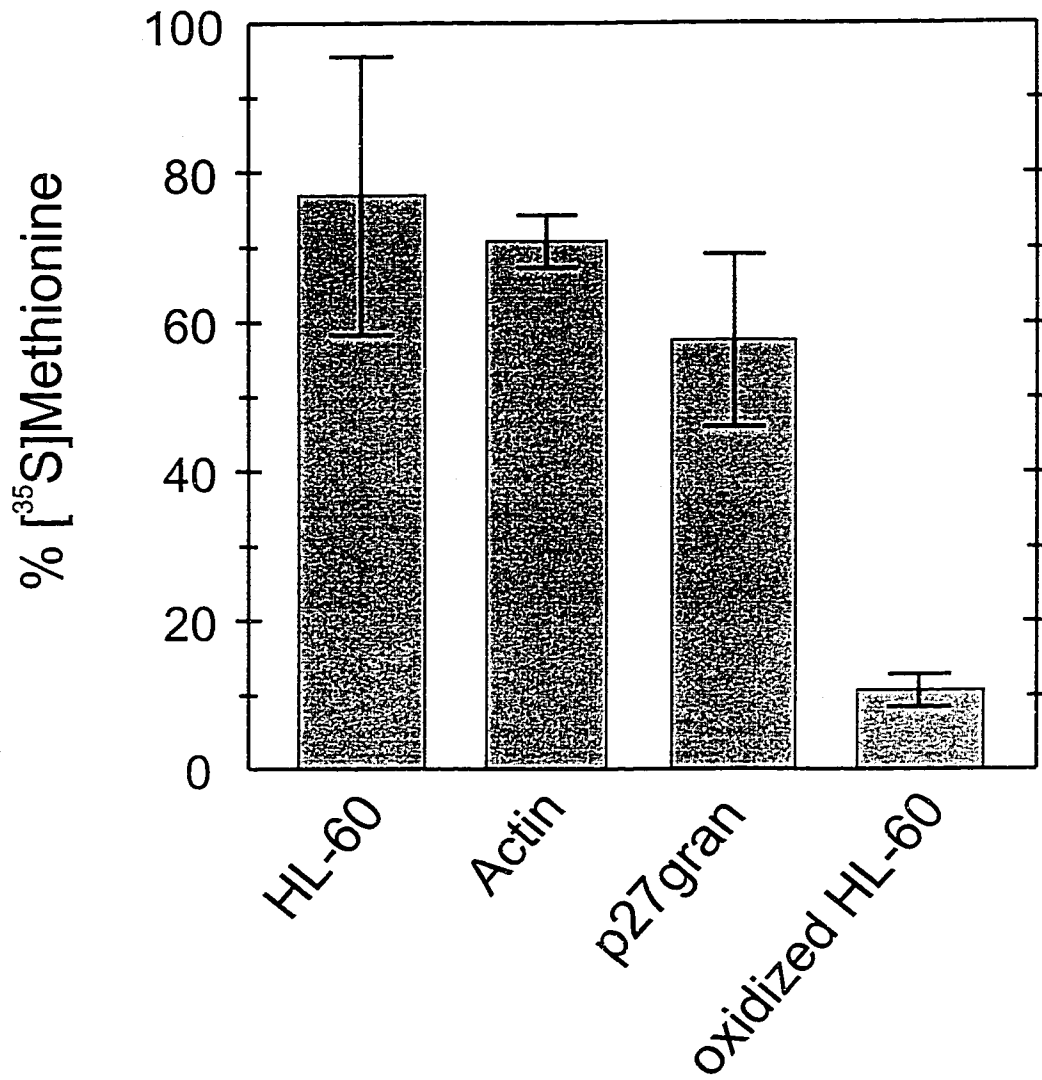
To verify that this unknown was indeed a protein, granulocyte extracts were digested with a protease prior to electrophoresis. As shown in Figure 5.2 lane 6, proteinase K degraded all bands on the gel, including p27gran. Pretreatment of granulocytes with actinomycin D (an RNA synthesis inhibitor) failed to abrogate the relative increase of the ^{35}S -labeled p27gran (compare Figure 5.2, lanes 2 and 4). Therefore, increased radiolabeling of this unknown protein required no new RNA synthesis and likely occurred post-transcriptionally. Actinomycin D did reduce the amount of [^{35}S]Met incorporated into the cells by ~45% likely because mRNA levels would drop during the 4 h treatment. Pretreatment of granulocytes with the protein synthesis inhibitor cycloheximide reduced incorporated radiolabel by ~70%. Cycloheximide did inhibit translation of the unknown

protein since $\sim 3.5\times$ more protein had to be loaded in lane 5 of Figure 5.2 to standardize incorporated cpm's. However, when compared to other proteins (e.g., β/γ -actin), p27gran was appreciably less affected by cycloheximide (Figure 5.2, lane 5).

The reason that cycloheximide inhibited radiolabeling of p27gran to a lesser extent than β/γ -actin was investigated further. Mitochondrial translation is not affected by cycloheximide, but is inhibited by chloramphenicol [289]. Pretreatment of granulocytes with 10 $\mu\text{g/mL}$ chloramphenicol had no effect on radiolabeling of p27gran (data not shown). Therefore, the unknown band was not a protein encoded by the mitochondrial genome, but this was not surprising since neutrophils contain very few mitochondria (20-30 per cell) [1]. Also, minor contaminants in the commercial preparation of [^{35}S]Met may have contributed to post-translational radiolabeling of the unknown. For example, if contaminating [^{35}S]sulfate was present, it could become incorporated post-translationally into a sulfated glycoprotein. To verify that p27gran was indeed radiolabeled with [^{35}S]Met, the assay of Fliss *et al.* was used [290]. In this assay, cyanogen bromide specifically reacts with [^{35}S]Met to produce volatile methyl[^{35}S]thiocyanate that can be removed by evaporation. In my hands, [^{35}S]Met-labeled total HL-60 proteins demonstrated $\sim 80\%$ [^{35}S]Met by this assay (Figure 5.3, HL-60 bar), compared with 85% obtained by Fliss *et al.* [290]. Oxidation of [^{35}S]Met to [^{35}S]methionine sulfoxide renders it nonreactive to cyanogen bromide, demonstrating the specificity of the reaction (Figure 5.3, oxidized HL-60 bar). When both radiolabeled actin and p27gran from human granulocytes were purified on SDS-PAGE and subjected to the cyanogen bromide assay, values of $\sim 70\%$ and $\sim 60\%$ [^{35}S]Met respectively were obtained (Figure 5.3). These values were not statistically different from that of total HL-60 proteins.

Figure 5.3. Cyanogen bromide assay to determine the amount of [^{35}S]methionine in radiolabeled proteins. The assay was performed as described by Fliss *et al.* [290]. Actin and p27gran were purified on SDS-PAGE from [^{35}S]Met-labeled, GM-CSF-treated granulocytes as described in section 5.3.3. [^{35}S]Met-labeled HL-60 proteins were prepared by boiling in 10% TCA/0.2 M methionine and were oxidized with H_2O_2 as described previously [290]. Bars depict the mean $\pm$ SD ($n = 4$). The relatively large error bars with HL-60 and p27gran samples were caused by one low point which did not warrant omission. The means for both HL-60 and p27gran would have increased by $\sim 5\%$ had these "outliers" been discarded.

-111-
Figure 5.3



Therefore, there seems to be nothing unusual regarding the state of radiolabel of p27gran. The reason why translation of this protein shows relative resistance to cycloheximide is not known, but will be discussed in section 5.5.2.

One additional property of p27gran was demonstrated on SDS-PAGE. Radiolabeled granulocyte extracts were usually prepared by boiling cells directly in SDS sample buffer. This technique quickly inactivated endogenous proteases to maintain the integrity of the radiolabeled proteins. When cells were lysed in SDS sample buffer but only heated to 65°C, endogenous neutrophil proteases remained active and completely degraded proteins such as β/γ -actin. However, p27gran remained relatively intact (data not shown). Therefore, this unknown protein demonstrated resistance to digestion by endogenous neutrophil proteases. The protein can, however, be digested by exogenously added V8 protease as described in section 5.4.4.1.2.

5.4.4 - Attempt to identify p27gran

5.4.4.1 - Candidate protein approach

I first attempted to identify the unknown upregulated protein by simply testing candidate proteins. The criteria for a candidate were as follows: (i) It must have a deduced molecular weight of ~26-27 kDa, (ii) it must contain Met residues and (iii) it must be of relative abundance intracellularly.

5.4.4.1.1 - Hsp27 and p27Kip1

The effect of GM-CSF treatment on the levels of both Hsp27 and p27Kip1 were tested by immunoblotting. Polyclonal antisera to heat shock protein Hsp27 was obtained

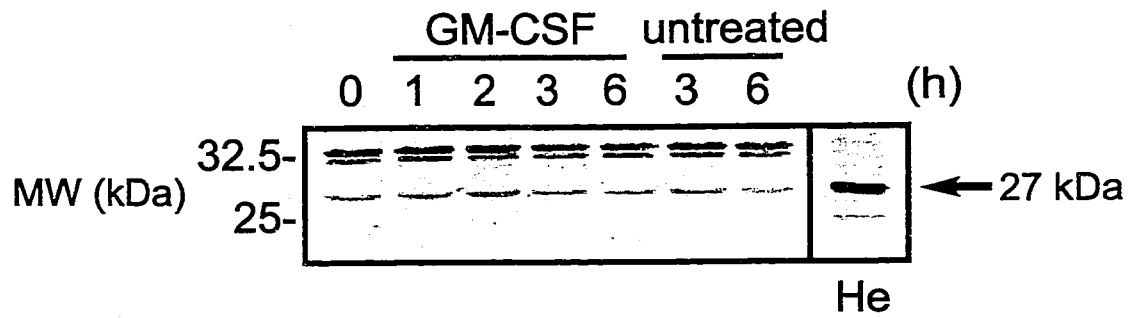
from Dr. Jacques Landry (Centre de recherche en cancerologie, Université Laval). This antibody readily detected a 27 kDa protein from HeLa cell extracts (Figure 5.4, lane He). A polyclonal antibody to the cell cycle regulator p27Kip1 was provided by Dr. Christine Pratt (Dept. of Pharmacology, University of Ottawa). The avidity of this antibody has been demonstrated previously [291]. Both antisera detected only tiny quantities of protein from granulocyte extracts (the granulocyte blots in Figure 5.4 have been overexposed), and the low levels that were detected showed no upregulation with GM-CSF treatment. Therefore, both Hsp27 and p27Kip1 were unlikely to be the unknown p27gran protein.

5.4.4.1.2 - hTegt

As shown previously in Chapter 4, granulocytes express relatively abundant quantities of hTegt mRNA. Analysis of the open reading frame of hTegt predicted a protein of 26.5 kDa containing 5.1 % Met residues, making hTegt a candidate for p27gran. Unfortunately, there was no antibody available to detect hTegt on Western blots. Other methods had to be utilized. The predicted sequence of hTegt revealed a highly hydrophobic protein, likely to be an integral membrane protein [292,293] (also see Swiss-Prot accession #P55061). Triton X-114 is a nonionic detergent of the Triton family which has a cloud point of 22°C [294]. X-114 dissolves in water below 22°C, but separates into a distinct phase upon heating above the cloud point. Membrane proteins either extract into the X-114 phase or remain in an insoluble fraction if they are associated with insoluble cellular components [286]. When [³⁵S]Met-labeled granulocytes were extracted with Triton X-114, p27gran remained in the aqueous phase (Figure 5.5A, lane 4). This suggested that the upregulated unknown was not a membrane protein and was not hTegt.

Figure 5.4. Western blotting of granulocyte proteins to detect potential candidates of p27gran. Freshly isolated granulocytes (0 time point) were cultured for the indicated amount of time (in hours) with (GM-CSF lanes) or without (untreated lanes) 20 ng/mL GM-CSF. Extracts were prepared by boiling cells for 5 min directly in SDS/Mops buffer. 30 µg total protein was loaded per lane of a 12% SDS-PAGE gel and Western blotting carried out as described in Chapter 2. *Hsp27*. Rabbit polyclonal antisera specific to heat shock protein 27 was used at 1/2000 dilution (obtained from Dr. Jacques Landry). *p27Kip1*. Rabbit polyclonal antisera specific to the cell cycle regulator p27Kip1 was used at 1/500 dilution. He, HeLa cell extract (10 µg total protein). The expected position of each candidate protein is indicated by an *arrow* at the right of each blot.

Hsp27



p27Kip1

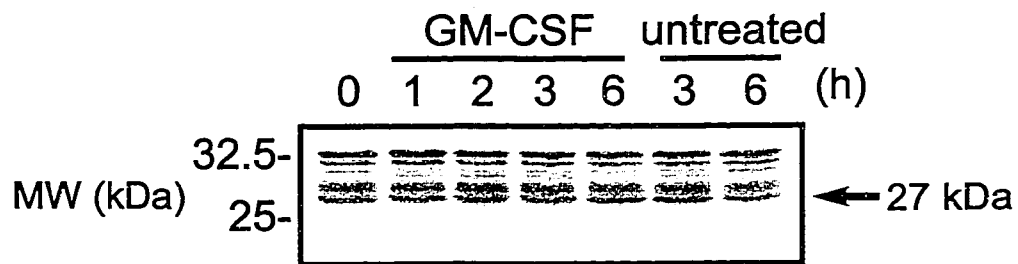
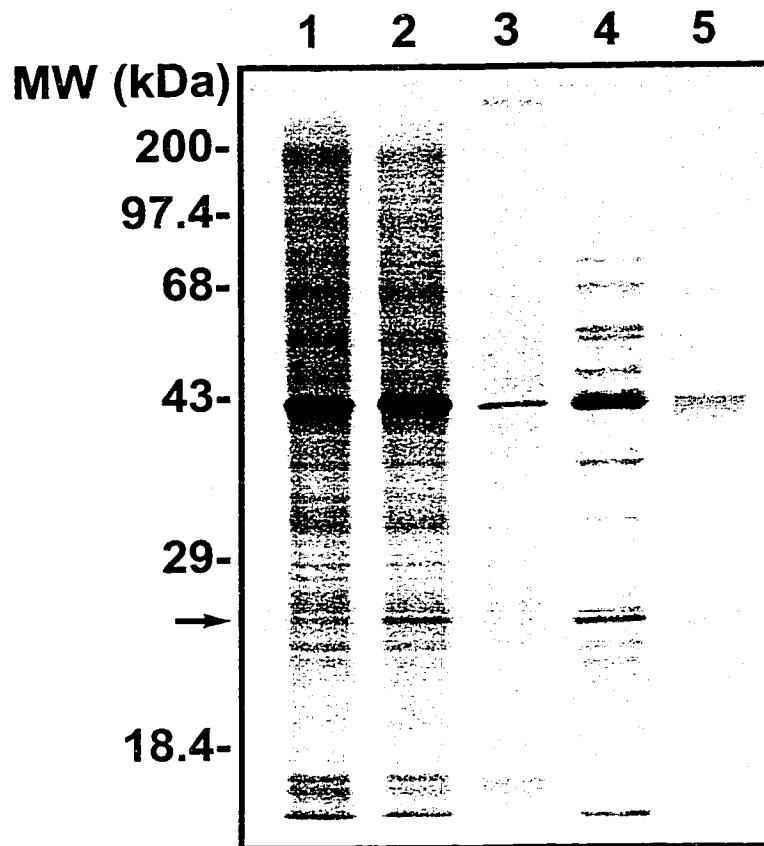
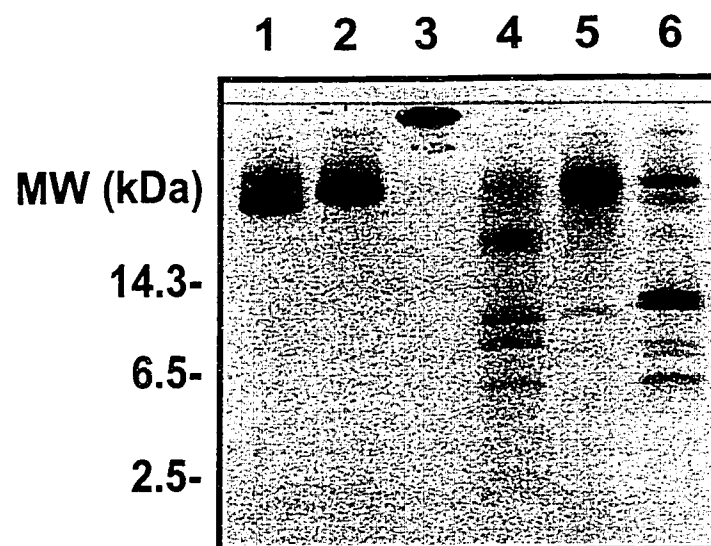


Figure 5.5. **The p27gran protein is not hTegt.** Both panels depict fluorograms of [³⁵S]Met-labeled proteins electrophoresed on SDS-PAGE. Panel A. Triton X-114 extraction. Granulocytes were radiolabeled for 5 h with 10 μ Ci/mL L-[³⁵S]Met and extracts electrophoresed as described in section 5.3.1. *Lane 1.* Total radiolabeled protein from granulocytes incubated without GM-CSF. *Lane 2.* Total radiolabeled protein from granulocytes incubated with 20 ng/mL GM-CSF. GM-CSF-treated granulocytes were subjected to Triton X-114 extraction as described in section 5.3.2. *Lane 3.* insoluble fraction, *Lane 4.* aqueous fraction, *Lane 5.* Triton X-114 fraction. The arrow depicts the position of p27gran. Panel B. V8 protease peptide mapping. The p27gran protein was purified on SDS-PAGE from radiolabeled, GM-CSF-treated granulocytes as described in section 5.3.3. *In vitro* translation was carried out as described in Chapter 2 to synthesize [³⁵S]Met-labeled hTegt and firefly luciferase, which were also purified on SDS-PAGE. Purified proteins were digested with endoproteinase Glu-C (V8 protease) (70 μ g/mL) for 2.5 h at 37°C in 0.1% SDS, 2 mM DTT, 50 mM sodium phosphate (pH 7.8). Extracts were electrophoresed on trilayered Tricine SDS-PAGE as described previously by Schägger and von Jagow [295] (4% T, 3% C stacking gel - 10% T, 3% C spacer gel - 16.5% T, 6% C resolving gel with glycerol). *Lane 1.* p27gran, *Lane 2.* *in vitro* translated hTegt, *Lane 3.* *in vitro* translated luciferase, *Lane 4.* p27gran digested with V8 protease, *Lane 5.* *in vitro* translated hTegt digested with V8 protease, *Lane 6.* *in vitro* translated luciferase digested with V8 protease. Molecular weight markers are as follows: lysozyme (14.3 kDa), aprotinin (6.5 kDa), and insulin chain A (2.5 kDa).

A



B

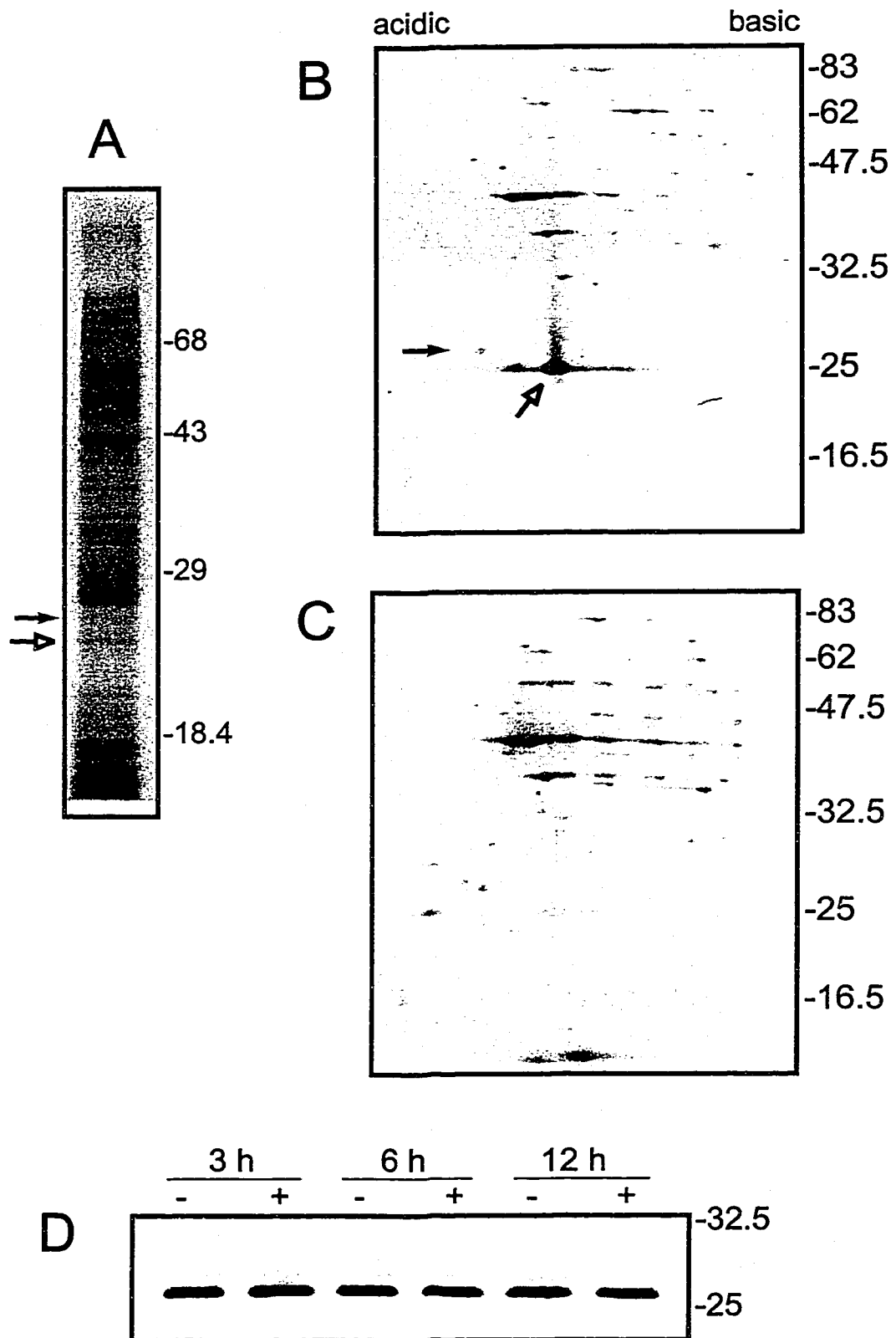


To further verify that the unknown was not hTegt, a cloned cDNA was obtained from the I.M.A.G.E. Consortium (#207657) which was verified to be the small transcript of hTegt by sequencing (see Chapter 6, section 6.3.1). Using this template, *in vitro* transcription and translation were carried out to produce [³⁵S]Met-labeled hTegt. *In vitro* translated hTegt, *in vitro* translated luciferase and p27gran were purified on SDS-PAGE. These proteins were digested with V8 protease and electrophoresed on discontinuous Tricine-SDS-PAGE [295] to produce peptide maps (Figure 5.5B). V8 digestion of p27gran produced discrete peptide fragments (Figure 5.5B, lane 4), while *in vitro* translated hTegt was almost completely resistant to digestion with V8 protease (Figure 5.5B, lane 5). Therefore, the unknown p27gran protein was determined not to be hTegt by both V8 peptide mapping and partitioning with Triton X-114 detergent. hTegt will be analyzed in greater detail in Chapter 6.

5.4.4.2 - Protein sequencing

Polyacrylamide gels of radiolabeled granulocyte proteins were usually stained with Coomassie blue prior to autoradiography to visualize molecular weight standards. Careful alignment of the Coomassie blue-stained gel with the autorad revealed that the p27gran band overlapped with a Coomassie blue-stained protein. This protein is indicated by a closed arrow in the Coomassie blue-stained gel shown in Figure 5.6A. [³⁵S]Met-labeled granulocyte extracts were electrophoresed on SDS-PAGE, taking appropriate precautions for protein sequencing as described in section 5.3.1. The gel was transferred to PVDF membrane, stained with Coomassie blue and exposed to x-ray film. The Coomassie blue-stained band

Figure 5.6. **The p27gran protein is not Gst π .** Panel A. Coomassie blue-stained 12% SDS-PAGE gel, loaded with 8×10^5 cell equivalents of protein. The position of p27gran (*closed arrow*) and the putative position of Gst π (*open arrow*) are shown. Panel B. Western blotting of 2-D polyacrylamide gel. [^{35}S]Met-labeled ($10 \mu\text{Ci/mL}$ for 5 h), GM-CSF-treated granulocytes were subjected to 2-D gel electrophoresis by the method of O'Farrell [297], following the instructions supplied with the instrument (Bio Rad Protean II electrophoresis system). The ampholyte mixture used in the first dimension was Pharmalyte 3-10:Bio-Lyte 5/7 (1:1). A 12% SDS-PAGE discontinuous gel was used in the second dimension. The 2-D gel was transferred electrophoretically to PVDF membrane and immunoblotted with 1/1000 diluted rabbit polyclonal Gst π antisera as described in Chapter 2. The *open arrow* depicts the position of the immunoreactive Gst π . A *closed arrow* shows the expected vertical position of p27gran. Panel C. Direct autoradiography of membrane in *panel B*. Prior to Western blotting, the membrane in *panel B* was exposed to a PhosphorImager screen to detect the position of radiolabeled protein. Panel D. Time-course of Gst π protein levels in GM-CSF treated and untreated granulocytes. Cells were cultured for the indicated amount of time in the presence (+) or absence (-) of 20 ng/mL GM-CSF. Soluble extracts were made by treating the cells with 0.5% Triton X-100, 1 mM CDTA, 1 mM PMSF, 2 $\mu\text{g/mL}$ aprotinin and 20 mM Mops/NaOH (pH 7.2) on ice for 20 min, then removing debris by microcentrifugation at 4°C. 5 μg total protein was loaded per lane of a 12% SDS-PAGE gel and Western blotting carried out as described in Chapter 2 with the Gst π antisera used in *panel B* above. Molecular weight standards are shown in kDa to the right of each panel.



that corresponded to the upregulated radiolabeled protein was cut from the membrane and subjected to protein sequencing. An amino acid sequence was obtained (PPYTVVYFPVRG), which matched identically to amino acids 2-13 of human glutathione S-transferase isoform pi (Gst π) (Genbank accession #121746).

Human neutrophils are known to contain significant Gst activity [296]. However, the predicted amino acid sequence of Gst π revealed a molecular weight of 23.2 kDa, indicating that p27gran may not be Gst π . To provide greater resolution, [³⁵S]Met-labeled granulocyte proteins were separated by 2-D gel electrophoresis and subjected to immunoblotting with polyclonal Gst π antisera (generously provided by Dr. Gordon Kirby, University of Guelph). The immunoreactive spot is indicated by an open arrow in Figure 5.6B, electrophoresing with an apparent molecular weight of ~25 kDa and a slightly acidic pI (theoretical value of 5.5). Unfortunately, based on its mobility on 1-D SDS-PAGE, p27gran would migrate 0.5 cm higher in the second dimension (Figure 5.6B, closed arrow). The exact position of p27gran during IEF is not known and will be discussed in section 5.5.2. Granulocyte extracts contained large quantities of Gst π by immunoblotting (signals developed within seconds), but only small quantities of [³⁵S]Met-labeled Gst π (Figure 5.6C). Finally, immunoblotting of granulocyte extracts on 1-D SDS-PAGE revealed that Gst π was not upregulated by GM-CSF even after 12 h of stimulation (Figure 5.6D). Therefore, I concluded that p27gran was not Gst π . Possible reasons as to why the protein sequence was spurious will be discussed below.

5.5 - Discussion

5.5.1 - Bcl-2 family members

Mature human granulocytes express the pro-apoptotic Bax protein, but express little or no anti-apoptotic Bcl-2, Bcl-X_L or Mcl-1 proteins. Stimulation with GM-CSF had no effect on the relative levels of these Bcl-2 family members; Bax was still predominant even after 24 h of GM-CSF treatment. That Bcl-2 expression is not upregulated by GM-CSF stimulation was confirmed recently by other investigators [16]. Another proinflammatory cytokine that delays the onset of apoptosis in human neutrophils, G-CSF, also does not induce expression of Bcl-2 or Bcl-X (unpublished data mentioned in [93]). It appears that mature neutrophils are not capable of expressing adequate levels of anti-apoptotic Bcl-2-related proteins. Other family members (e.g., Bik, Bak and Bad) were not assessed in this chapter because antibodies were not available at the time of study. However, since they are pro-apoptotic Bcl-2 family members, their expression may be irrelevant, since neutrophils express insufficient levels of the anti-apoptotic members. The high levels of Bax are consistent with the rapid onset of programmed cell death in circulating neutrophils. Neutrophils contain very few mitochondria (20-30 per cell) [1]. Bax expression alone can induce cell death most likely by opening large 'megapores' in mitochondria (mitochondrial permeability transition), allowing the release of cytochrome c and AIF. AIF and cytochrome c can then activate caspases of apoptotic pathways [281,298]. It is believed that neutrophils contain few mitochondria because they rely heavily on glycolytic catabolism for energy requirements [1]. It is possible that large numbers of mitochondria cannot exist in neutrophils because high Bax levels would induce rapid cell death that could not be

modulated by cytokines. However, the role of mitochondrial permeability transition in cell death is still a matter of dispute.

If the delay in apoptosis with cytokine-treatment does not occur via the Bcl-2 pathway, then another pathway(s) must be involved. Neutrophils express Fas (CD95), membrane-bound Fas ligand, as well as soluble Fas ligand that is released from cells when aged *in vitro*. Fas antagonistic antibodies reduce spontaneous neutrophil apoptosis in 72 h culture by ~50%, suggesting that the Fas system is an important, but not unique pathway leading to neutrophil death [93,94]. Cytokines such as GM-CSF and G-CSF can reduce Fas-mediated neutrophil apoptosis initiated with an agonistic anti-Fas IgM [93]. Therefore, cytokine treatment may induce the transcription/translation of a factor that is inhibitory to the Fas system. No such factor has yet been reported. To my knowledge, other factors known to interact with Fas, such as Fadd, Rip, Fap-1 and Flip (also called Casper), have not been identified in granulocytes [94,299,300]. The mechanism by which Fas induces programmed death in these cells warrants further analysis, including the possible involvement of ROS as second messengers [92,101].

5.5.2 - p27gran protein

An unidentified 26-27 kDa granulocyte protein (p27gran) demonstrated a 2-3-fold increase in [³⁵S]Met-labeling on SDS-PAGE with GM-CSF treatment. In my hands, this protein showed the largest and most consistent increase of all bands resolved on a 12% gel. Waksman *et al.* demonstrated increased radiolabeling of 10 proteins on SDS-PAGE, including two unidentified proteins of 28 and 26 kDa. Either of these proteins could be

p27gran. Waksman *et al.* also identified a 37 kDa CAP as the species demonstrating the largest increase in radiolabeling with GM-CSF treatment. The reason why I did not observe a similar effect is not known, but could be due to different culturing conditions. Waksman *et al.* cultured neutrophils in serum-free media at higher concentrations ($2-5 \times 10^6/\text{mL}$) [278], while I used media containing 10% autologous serum at a cell concentration of $1 \times 10^6/\text{mL}$. Serum factors likely played a role, but their inclusion more closely mimics the *in vivo* milieu. Rather than load equal amounts of protein, I routinely loaded equal amounts of radioactivity between gel lanes. This allowed the identification of relative rather than absolute differences in radiolabeling and facilitated interpretation of the inhibitor study shown in Figure 5.2.

The upregulation of p27gran could not be prevented by inhibition of RNA synthesis, strongly suggesting post-transcriptional regulation. A similar conclusion was drawn by Waksman *et al.* [278] regarding radiolabeled protein synthesis in their GM-CSF-stimulated neutrophils. The increase in radiolabeling observed with p27gran was caused most likely by a direct effect on translation. However, the translational regulation was limited to certain mRNAs; not all proteins showed increased radiolabeling with GM-CSF. The fact that cycloheximide inhibited translation of p27gran less than that of β/γ -actin is peculiar, but may be due to a particular feature of neutrophils. These cells have few ribosomes and little ER [1,38]. β/γ -actin is synthesized on cytosolic ribosomes, but if p27gran were destined for secretion or granule storage, it would have to dock on the ER before translation could be completed. Cycloheximide exerts its inhibition directly on ribosome function [301]. If ER docking sites are the limiting factor in neutrophils, a model to explain my observation is given in Table 5.I. In this example, cycloheximide would cause an apparent reduction in

actin and p27gran translation of 95% and 50% respectively, although this example is slightly exaggerated for emphasis.

Table 5.I
Hypothetical example of the effect of cycloheximide on apparent protein synthesis

<u>Protein</u>	<u>Ribosome synthetic capacity (molecule/s)</u>	<u>ER docking capacity (molecule/s)</u>	<u>Apparent protein synthesis (molecule/s)</u>	<u>Apparent % inhibition of protein synthesis</u>
Actin	20	2	20	
Actin + cycloheximide	1	2	1	95
p27gran	20	2	2	
p27gran + cycloheximide	1	2	1	50

The hypothetical example shown in Table 5.I assumes that p27gran must dock on the ER to be synthesized. Consistent with this assumption is the observation that p27gran was resistant to degradation by endogenous neutrophil proteases. Neutrophilic secretory granules contain metalloproteases and neutral proteases, such as cathepsin G and elastase [302]. Proteins that are packaged into secretory vesicles must be resistant to proteolytic digestion in order to remain functional. Interestingly, site-directed mutagenesis has demonstrated that cathepsin G has a molecular weight of 27.5 kDa on SDS-PAGE if its C-terminal prodomain is removed but N-linked glycosylation does not occur [303]. Cathepsin G could be a potential candidate for p27gran since mature neutrophils post-translationally process some

proteins slowly (e.g., Fc receptor) [204]. However, cathepsin G is packaged exclusively in azurophilic granules which are synthesized early in neutrophil differentiation and not in mature cells [302].

Several attempts were made to identify p27gran, but none were successful. It was recognized that the chance of identifying this unknown protein was low, considering that there are numerous proteins of the expected size, many of which have yet to be cloned. However, a few candidates were tested. Using Western blotting, both p27Kip1, a cell cycle regulator thought to cause G1-arrest in hematopoietic cells [304], and the ubiquitously expressed Hsp27 were ruled out as potential candidates. Also, hTeg encoded by a relatively abundant mRNA in granulocytes was eliminated as a candidate. The radiolabeled band was found to overlap a lightly-staining protein on Coomassie blue-stained gels. Since 1-D SDS-PAGE does not produce high resolution separation, overlap does not necessarily equate the two proteins. However, I had already concluded that p27gran likely represented increased translation of an already abundant species. Therefore, the Coomassie blue-stained band was a possible candidate. Peptide sequencing revealed the band to be human Gst π , but further analysis determined that p27gran could not be Gst. On 1-D SDS-PAGE, p27gran migrated 0.5 cm more slowly (Figure 5.6A, closed arrow) than the predicted migration of Gst π (Figure 5.6A, open arrow). Although it is possible that the wrong band was subjected to protein sequencing, a more likely explanation is that p27gran was blocked at its N-terminus and was refractory to Edman degradation. Upwardly smearing Gst π (see Figure 5.6B) that was not blocked at its N-terminus would be sequenced as a contaminant. In support of this hypothesis, the amount of protein sequenced was lower than the amount of protein estimated

by Coomassie blue staining (Dr. Yaguchi, personal communication).

Although I was not able to complete my study of p27gran, further progress would require fragmentation of the protein with cyanogen bromide, and separation of the peptides by chromatography. Cyanogen bromide-treatment will generate free N-termini in the cleaved peptides, allowing them to be sequenced. Initial purification of the protein on 2-D gels rather than 1-D SDS-PAGE would also be useful. Waksman *et al.* [278] concluded that many of the radiolabeled proteins upregulated with GM-CSF were cationic, although the authors concentrated on 37- and 57-kDa species. If p27gran is cationic, it would not resolve on a standard IEF gel; special precautions would have to be taken to resolve very basic or acidic proteins [305].

In summary, the following properties of p27gran have been demonstrated. (i) It is a protein (sensitive to proteinase K and V8 but resistant to endogenous neutrophil proteases) that migrated with an apparent molecular weight of 26-27 kDa on SDS-PAGE. (ii) In a 3-6 h radiolabeling with [³⁵S]Met, the protein demonstrated a 2- to 3-fold increase in percentage of total cell-associated radiolabel (relative increase) following stimulation with 20 ng/mL GM-CSF; the absolute increase in radiolabeling was 3- to 4-fold. (iii) The increase in radiolabeling occurred post-transcriptionally, possibly at the level of translation. (iv) Radiolabeling of p27gran was inhibited by cycloheximide to a lesser extent than other proteins. I have postulated that this occurred because its synthesis involved docking on the ER. However, the subcellular localization of p27gran has yet to be determined. (v) The unknown was not Hsp27, p27Kip1, hTegt, Gst π , Bcl-2 or Bcl-X_L. It was also not encoded by the mitochondrial genome. (vi) p27gran was likely an abundant protein in human

granulocytes. (vii) p27^{gran} is likely a nonmembrane protein since it did not extract into Triton X-114 during phase separation.

Chapter 6

**Characterizing the properties of Tegt, a protein of unknown function
that is expressed in human granulocytes.**

6.1 - Summary

Human testis-enhanced gene transcript (hTegt) has been studied previously at the nucleic acid level [292,293]. Although the mRNA is conserved and expressed in many cells including human granulocytes, the protein has no known function because the predicted amino acid sequence lacks consensus domains. This chapter details the study of hTegt at the protein level. My attempts to raise polyclonal antibodies against a synthetic peptide corresponding to the C-terminus of hTegt were unsuccessful. High serum titres were obtained, but the antisera did not immunoprecipitate hTegt nor did it detect the protein on Western blots. As an alternative, the full-length hTegt was obtained from the I.M.A.G.E. consortium [245] and used to construct mammalian expression plasmids. The predicted amino acid sequence of the I.M.A.G.E. clone (GenBank accession #AF033095) was identical to that of Walter *et al.* (GenBank accession #X75861) with the exception of 2 substitutions (Pro¹⁶⁹→Leu¹⁶⁹ and Val¹⁸⁷→Phe¹⁸⁷). These substitutions make the hTegt protein identical to that of the rat at amino acids 169 and 187, rather than different as was originally thought. Various conclusions were drawn about hTegt using *in vitro* translation and transient transfections of both full-length and HA-tagged protein. (i) The protein demonstrated no obvious post-translational modifications such as signal-peptide cleavage, N-linked glycosylation or O-linked glycosylation. (ii) The protein retained some secondary and tertiary structure in the presence of SDS. When boiled in SDS, it aggregated homotypically or heterotypically, a property believed to be caused by its high hydrophobicity. (iii) hTegt and HA-tagged hTegt were toxic after 24 h when overexpressed in 293T cells. (iv) *In vitro* translation of the protein in reticulocyte lysates required the presence of microsomes for

efficient synthesis, suggesting that hTegt targets to the ER. Immunofluorescence of HeLa cells transiently expressing an HA-tagged hTegt also localized the protein to the ER and contiguous structures such as the nuclear membrane. Although the function of this protein still remains a mystery, important information regarding its properties has been gained that should be helpful in any future analyses.

6.2 - Introduction

In 1994, Walter *et al.* serendipitously cloned a novel cDNA from a rat testis expression library that they termed testis-enhanced gene transcript (Tegt) [292]. The authors studied this gene at the nucleic acid level in rat, mouse and human [292,293]. A summary of what is known of Tegt from the work of Walter *et al.* follows. (i) Two transcripts of 2.8 and 1.0 kb show broad tissue expression [306]. Both transcripts are derived from the same gene due to differential polyadenylation, and both contain the full ORF. Both transcripts are of approximately equal abundance in most tissues with the exception of rat and mouse testes, where the smaller transcript is ~5-fold more abundant. Accumulation of the small transcript coincides with protamine synthesis, indicating a postmeiotic differential expression. Enhanced small transcript expression is not observed in human testes. (ii) "Zoo" blots indicate that the single-copy Tegt gene is highly conserved. (iii) Rat Tegt (rTegt) maps to chromosome 7, mouse Tegt (mTegt) to chromosome 15, and human Tegt (hTegt) to chromosome 12q12-q13. These regions belong to a syntenic group that is conserved in rat, mouse and human. (iv) The deduced amino acid sequence of Tegt predicts a highly hydrophobic protein of 236 amino acids in rat and 237 amino acids (26473 Da) in human.

Between rat and human, the coding region of Tegt demonstrates 85% nucleotide and 90% amino acid similarity indicating a highly conserved protein. Seven potential transmembrane domains exist in the predicted amino acid sequence (Swiss-Prot accession #P55061). (v) The deduced amino acid sequence is not homologous to other known vertebrate sequences. However, it does show a low but significant homology to the hypothetical ycca protein from *E. coli* (23.5 kDa, 27% identity, Swiss-Prot accession #P06967) [307], *Pseudomonas aeruginosa* (24.0 kDa, 25% identity, Swiss-Prot accession #Q03268) [308] and *Haemophilus influenza* (24 kDa, Swiss-Prot accession #P44477). All five proteins (hTegt, rTegt and the 3 ycca members) have been grouped as a family of small, integral membrane proteins under Prosite ID #UPF0005. The function of this family of proteins is not known currently. (vi) Walter *et al.* found no consensus sequences in the predicted amino acids with the exception of a potential N-linked glycosylation site at position 110 (N¹¹⁰P¹¹¹S¹¹² in human), and a potential bipartite NLS near the C-terminus (R²¹⁹K²²⁰...D²³¹K²³²K²³³K²³⁴E²³⁵ in human) [309]. However, it is questionable whether these consensus sequences are functional. In the N-linked glycosylation consensus sequence (NXS/T) X is not commonly a proline residue [310]. Also, assuming that Tegt is an integral membrane protein, targeting to the nucleus is unlikely.

Tegt is predicted to be a conserved protein with a strong and ubiquitous tissue expression. This suggests that the protein serves an important function in many mammalian cells including human granulocytes. Analysis of the predicted amino acid sequence reveals little about the function of Tegt since no definitive consensus sequences are present. Despite the observation that hTegt mRNA levels remained unchanged in human granulocytes with

GM-CSF-stimulation (see Chapter 4, Figure 4.5D), the protein was studied further to gain insight into its function. The limited life-span and low transcription/translation rates of neutrophils make manipulations such as transient and stable transfections difficult (see Chapter 1). Because such molecular biology techniques were required to further study hTeg, the experiments in this chapter utilized cultured human cell lines (i.e., 293T, HeLa and HL-60). More specifically, techniques such as transient transfections of epitope-tagged constructs, Western blotting, cellular immunofluorescence, *in vitro* transcription/translation and raising of polyclonal antisera were used. Although the function of hTeg in mammalian cells could not be determined, several important properties of the protein were established and presented in this chapter. An appreciation of these properties may be instrumental in any future studies.

6.3 - Materials and Methods

6.3.1 - Oligonucleotide-directed mutagenesis of hTeg

Plasmid DNA from I.M.A.G.E. clone #207657 was electroporated into *E. coli* strain XL-1 Blue, and a single colony picked from LB agar + 100 µg/mL ampicillin. Single-stranded sense DNA was produced from this colony as follows. The colony was inoculated into 2 mL 2×YT broth containing 2×10^7 pfu/mL bacteriophage M13KO7, and was grown at 37°C with vigorous shaking until slightly turbid (~3 h). 70 µg/mL kanamycin was added and the incubation continued overnight. Bacteria were pelleted by microcentrifugation for 5 min at 4°C. Bacteriophage particles were precipitated from the supernatant by addition of 200 µL (20% polyethylene glycol 8000, 2.5 M NaCl) per 1.2 mL supernatant and

incubation at room temperature for 15 min. The mixture was microcentrifuged for 5 min at 4°C, and the supernatant discarded. Polyethylene glycol-precipitated phage were suspended in 100 µL (2 mM MgCl₂, 10 mM Tris/HCl (pH 7.5)), and were digested with 20 µg/mL crude pancreatic DNase at 37°C for 10 min. The mixture was extracted once with 50 µL CT-saturated phenol, extracted once with CHCl₃, and precipitated with 75% ethanol, 0.1 M sodium acetate (pH 5.2). The pellet was washed with 70% ethanol, dried and dissolved in CT. The yield of ssDNA was determined from its absorbance at 260 nm.

15 ng synthetic oligodeoxynucleotide #6.1 (Table 6.I, 5'-phosphorylated with T4 polynucleotide kinase and ATP) and 0.5 µg ss207657 were heated to 75°C in 1 × SSC, and then allowed to anneal by lowering the temperature to 37°C at a rate of 2°C per min. Double-stranded DNA was synthesized by adding T4 DNA polymerase (6.7 unit), T4 DNA ligase (1 unit), 0.5 mM each dNTP, 1 mM ATP, 2 mM DTT, 10 mM MgCl₂, and 50 mM Tris/HCl (pH 8.0). The reaction was incubated on ice for 5 min, at room temperature for 5 min, and then at 37°C for 2 h. The reaction was stopped by addition of CDTA to 15 mM. The dsDNA was concentrated by ethanol-precipitation, and electroporated into *E. coli* XL-1 Blue. Single colonies grown on LB agar + 100 µg/mL ampicillin were selected and mini-prep plasmid DNA isolated. Clones were screened by restriction mapping for the presence of the new *Spe*I site (mutant plasmid named hTegt-Spe).

6.3.2 - Construction of mammalian expression plasmids

Diagrammatic representations of the following 4 plasmids are shown in Figure 6.1.

Table 6.I

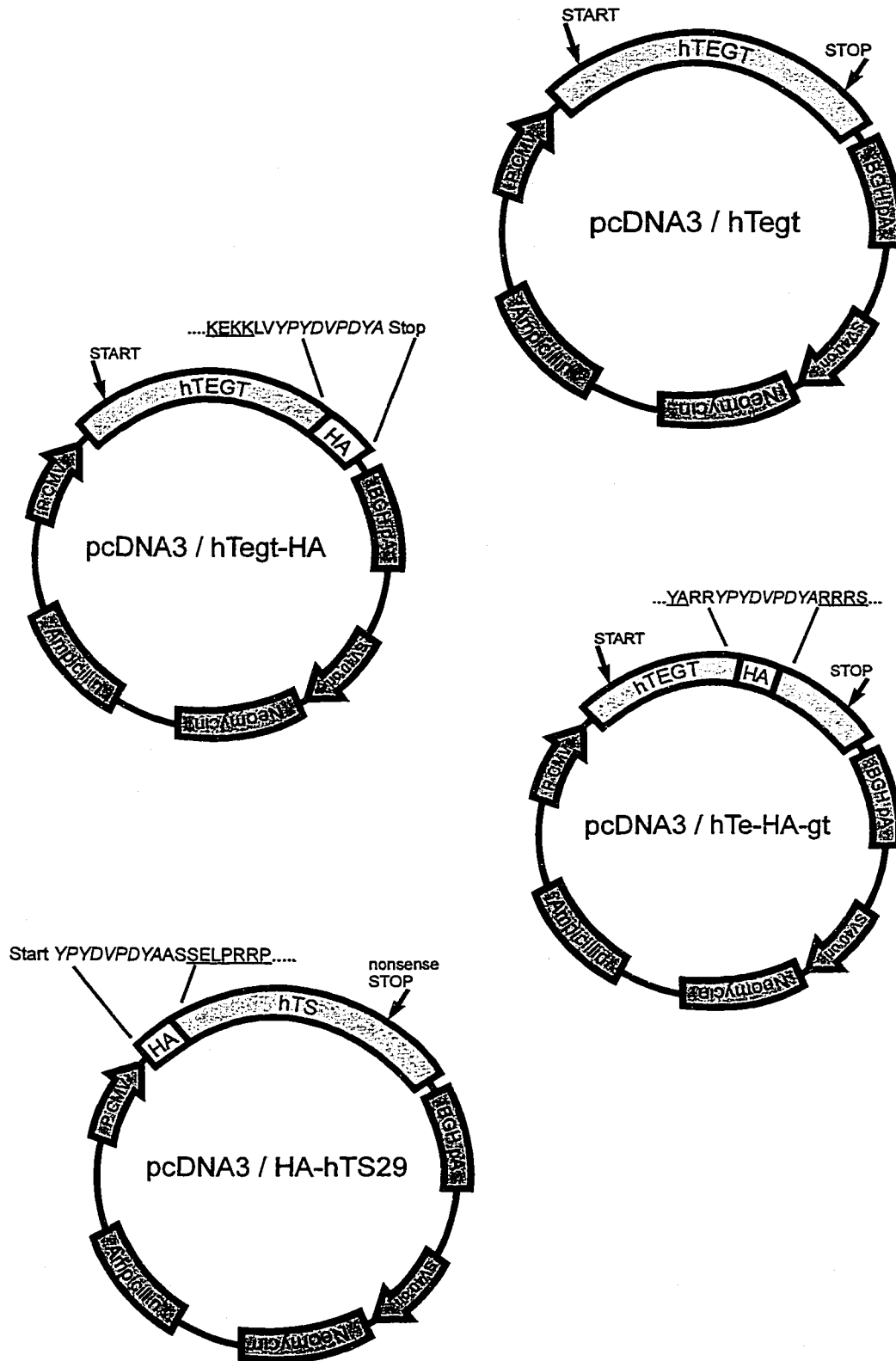
Synthetic oligodeoxynucleotides used to produce mammalian expression constructs

Oligodeoxynucleotides were synthesized by Life Technologies (Burlington, Ontario) at a 50 nmole scale and standard purity.

<u>Oligo ID</u> <u>number</u>	<u>Oligo</u> <u>length</u> <u>(bases)</u>	<u>Oligodeoxynucleotide sequence (5' – 3')</u>
6.1	27	GGATGGTCAACTAGTTTCTTCTCTTTC
6.2	36	CTAGTATATCCTTATGATGTCCCCGATTATGCCTGA
6.3	32	TCAGGCATAATCGGGGACATCATAAGGATATA
6.4	33	CGCTATCCTTATGATGTCCCCGATTATGCCAGG
6.5	33	CGCCTGGCATAATCGGGGACATCATAAGGATAG
6.6	35	AATTCATGTATCCTTATGATGTCCCCGATTATGCC
6.7	31	GGCATAATCGGGGACATCATAAGGATACATG
6.8	17	GCGAGCTCGGAACTGCC
6.9	21	GCACCCTAAACAGCCATTTC

Figure 6.1. **Mammalian expression constructs used in Chapter 6.** Diagrammatic representations of the 4 pcDNA3-derived constructs are depicted. Constructs were made as described in section 6.3.2. Regions of the pcDNA3 backbone are shown with dark grey shading, while those of the insert are shown with lighter grey shading. Transcription begins in the CMV promoter region (P CMV) and ends in the BGH polyadenylation sequence (BGH pA). Translation start and stop codons are highlighted. The expected amino acid sequence near the HA tag is shown expanded from the vector. In this region, the amino acids of the HA tag are *italicized*, and those endogenous to the protein are underlined. To simplify the figure, the *E. coli* origin of replication, the f1 origin of replication, the T7 promoter, the SP6 promoter, and the SV40 polyadenylation sequence have been omitted from the pcDNA3 backbones.

-133-
Figure 6.1



6.3.2.1 - pcDNA3 / hTegt

I.M.A.G.E. clone #207657 was digested with *EcoRI* and *NotI*. The resulting hTegt fragment (~900 bp, agarose gel purified) was ligated into *EcoRI*- and *NotI*-digested pcDNA3 with T4 DNA ligase.

6.3.2.2 - pcDNA3 / hTegt-HA

hTegt-Spe, generated by oligo-directed mutagenesis, was digested with *EcoRI* and *SpeI*, and the fragment (~800 bp) purified by agarose gel electrophoresis. To generate the HA-tag portion, synthetic oligodeoxynucleotides #6.2 and #6.3 (Table 6.I) were annealed by cooling in $2 \times$ SSC from 85°C to 4°C at a rate of 1°C per min. The hTegt-Spe fragment and synthetic HA-tag were ligated simultaneously into *EcoRI*- and *EcoRV*-digested pcDNA3 with T4 DNA ligase.

6.3.2.3 - pcDNA3 / hTe-HA-gt

I.M.A.G.E. clone #207657 was digested with *NarI*, *EcoRI* and *NotI*, and the 2 hTegt fragments (~480 and ~420 bp) purified on an agarose gel. To generate the HA-tag portion, synthetic oligodeoxynucleotides #6.4 and #6.5 (Table 6.I) were annealed by cooling in $2 \times$ SSC from 85°C to 4°C at a rate of 1°C per min. The hTegt fragments and synthetic HA-tag were ligated simultaneously into *EcoRI*- and *NotI*-digested pcDNA3 with T4 DNA ligase.

6.3.2.4 - pcDNA3 / HA-hTS29

The hTS open reading frame was amplified by PCR from the plasmid pUC19.TS4 (provided by Dr. Daniel Santi, UCSF) using *Taq* DNA polymerase (primers #6.8 and #6.9 from Table 6.I, 94°C/ 1 min, 65°C/ 1 min and 72°C/2 min). The 938 bp PCR product was blunted by treatment with T4 DNA Polymerase, 5'-phosphorylated by T4 polynucleotide

kinase, and purified by agarose gel electrophoresis. To generate the HA-tag portion, synthetic oligodeoxynucleotides #6.6 and #6.7 (Table 6.I) were annealed by cooling in $2 \times$ SSC from 85°C to 4°C at a rate of 1°C per min. The synthetic linker and PCR product were ligated into pcDNA3 that had been cut previously with *EcoRI* and *EcoRV*.

Competent XL-1 Blue, DH5 α or SURE *E. coli* were transformed with ligation mixtures, and were plated on LB agar + 100 $\mu\text{g/mL}$ ampicillin. Individual colonies were inoculated into $2 \times$ YT broth containing 100 $\mu\text{g/mL}$ ampicillin and were grown overnight at 37°C with vigorous shaking. Plasmid mini-preps were carried out on portions of the cultures and the desired constructs verified by restriction endonuclease mapping and/or sequencing.

6.3.3 - Production of polyclonal antisera

Amino acids 219-233 of the predicted hTegt sequence (RKLMMLAMNEKDKK) were synthesized as a tetrameric multiple antigenic peptide (hTegt MAP4) [311] by Ricardo Marius of the Cancer Research Group (Ottawa Regional Cancer Centre, Canada). Dried hTegt MAP4 was suspended in sterile PBS to a concentration of 10 mg/mL by extensive sonication (Sonifier Cell Disruptor 185, Branson Sonic Power Co., Conn.; solubility limit of peptide $\sim 1 \text{ mg/mL}$). The suspension was emulsified with an equal volume of Freund's complete adjuvant, and was injected intramuscularly into 6 female New Zealand white rabbits (1000 $\mu\text{g/rabbit}$). Rabbits were boosted every 4-6 weeks with hTegt MAP4 emulsified in Freund's incomplete adjuvant (500 $\mu\text{g/rabbit}$). Ten days after boosting, blood samples were collected and serum titre was quantified by ELISA analysis. Blood had also been drawn from the rabbits prior to any injection to serve as a preimmune serum control.

6.3.4 - ELISA for hTegt MAP4 antisera

96-well polystyrene plates were coated with 1 µg hTegt MAP4 synthetic peptide per well by adding the peptide in water and drying in a fumehood overnight. The wells were washed 3× with 0.05% Tween-20 in PBS (wash solution) to remove unbound peptide. The wells were blocked for 1 h at room temperature with 2% BSA in wash solution. Rabbit antisera or preimmune sera, diluted appropriately in wash solution, was allowed to bind to the wells for 1 h at 37°C. The wells were washed 3× with wash solution (room temperature, 3 min per wash). Alkaline phosphatase-labeled goat anti-rabbit IgG was allowed to bind to the wells for 1 h at 37°C (diluted 1/1000 in wash solution). Secondary antibody was washed from the wells as indicated above. 1 mg/mL p-nitrophenylphosphate, 0.5 mM MgCl₂, 10% ethanolamine (pH 9.8) was added to the wells and colour allowed to develop for 30 min at room temperature. The reaction was stopped by addition of CDTA to 50 mM, and the absorbance at 405 nm read using a microtitre plate reader. Background-corrected absorbance was calculated by subtracting the absorbance of preimmune sera from that obtained by the same dilution of antisera.

6.3.5 - Immunoprecipitation of [³⁵S]hTegt

[³⁵S]hTegt was synthesized by *in vitro* transcription and translation using wheat germ lysates and L-[³⁵S]methionine as described in Chapter 2. The *in vitro* translation reaction was diluted into 0.5% Triton X-100, 1 mM CDTA, 1 mM PMSF, 2 µg/mL aprotinin and 20 mM Mops/NaOH (pH 7.2) (wash buffer). The diluted reaction was allowed to stand on ice for 30 min, and was then clarified by microcentrifugation at 4°C for 20 min. Rabbit antisera or

preimmune sera was added to a final dilution of 1/2000, and the antibodies allowed to bind on ice for 60 min. Protein A-Sepharose was added (final concentration $\approx 15 \mu\text{g}/\mu\text{L}$) and the tubes mixed for 60 min at 4°C on a rotary mixer. The samples were microcentrifuged at room temperature for 10 s, and the supernatant discarded. The Sepharose beads were washed $3\times$ with wash buffer. $25 \mu\text{L}$ SDS sample buffer was added to the beads and heated at 65°C for 2 min. The supernatant was then subjected to liquid scintillation counting. To quantify the incorporation of radiolabel into hTeg, an aliquot of the *in vitro* translation reaction was precipitated with 25% TCA in the presence of 0.5% carrier BSA. The acetone-washed pellet was dissolved in 1% SDS, 0.1 M Tris/HCl (pH 7.5) and counted in a liquid scintillation counter.

6.3.6 - Immunofluorescence of cultured cells

HeLa cells were plated onto sterile glass coverslips in 6-well tissue culture plates at 3×10^4 cells per well in 2 mL DMEM + 10% FCS. The cells were allowed to grow for 24 h. Cells were then transfected with pcDNA3 constructs (Figure 6.1) using liposomes according to the manufacturer's instructions (Dodac:Dope LuV's, a gift from Inex Pharmaceuticals Corp., Vancouver, Canada). After a 5 h exposure of cells to liposomes, the media was replaced with DMEM + 10% FCS, and the incubation continued for a total of 72 h. Cells were washed $3\times$ with PBS, fixed for 10 min at room temperature with 4% paraformaldehyde in PBS (or 1:1 methanol:acetone for pcDNA3 / HA-hTS29 construct), and washed again. Coverslips were attached to microscope slides with nail polish. Coverslips were then treated sequentially with the following, at room temperature. (i) $100 \mu\text{L}$ 5%

normal goat serum, 1% BSA, 0.05% NP-40 in PBS (Ab dilution buffer [312]) for 15 min. (ii) 100 μ L 5 μ g/mL anti-HA monoclonal antibody in Ab dilution buffer for 2 h in a humidified chamber. (iii) Three washes (5 min each) of PBS. (iv) 100 μ L Texas Red-labeled goat anti-mouse IgG (1/500 in Ab dilution buffer) for 1 h in a humidified chamber. (v) Three washes (5 min each) of PBS. Slides were overlaid with antifade mounting media [313] and another coverslip. Cells were viewed by epifluorescence microscopy or by confocal fluorescence microscopy. Confocal microscopy was performed at the University of Ottawa Eye Institute Imaging Facility on an inverted LSM 410 confocal laser scanning microscope. Microscopes were manufactured by Carl Zeiss Inc. of Germany.

6.4 - Results

6.4.1 - Acquisition of a full-length hTegt cDNA and verification of its nucleotide sequence

To perform experiments with hTegt protein, a cDNA that contained the full coding region was needed. As mentioned in Chapter 4, a fragment of the hTegt sequence was originally cloned while performing DDRT-PCR on granulocyte RNA. This sequence did not encompass the entire ORF. Rather than search a cDNA library or use RT-PCR, a cDNA was obtained from the I.M.A.G.E. consortium [245]. Based on limited sequence information from both the 5'- and 3'-end (GenBank accession #H60355 and H59083), I.M.A.G.E. clone #207657 (derived from human fetal liver/spleen RNA) was likely the small transcript of hTegt. The clone was obtained and was sequenced. This sequence, shown in the upper line of Figure 6.2, matches precisely to the sequence of hTegt originally published by Walter *et al.* [293] (GenBank #X75861, Figure 6.2, lower line), except for two minor differences. (i)

Figure 6.2. **Comparison of the published sequence of the hTegt small transcript to that obtained by sequencing I.M.A.G.E. clone #207657.** The known sequence of hTegt small transcript (derived from Genbank accession #X75861, *lower sequence*) is compared to the automated sequence obtained from I.M.A.G.E. clone #207657 (*upper sequence*). Predicted start and stop codons are underlined. Shown in **BOLD** type are codons containing a sequencing difference, resulting in the indicated amino acid substitutions. Shaded regions depict the *NarI* restriction site and the *SpeI* restriction site (derived by oligo-directed mutagenesis) used to construct vectors pcDNA3 / hTe-HA-gt and pcDNA3 / hTegt-HA (see Figure 6.1) respectively. Nucleotide counts are indicated at the right of each sequence.

-139-
Figure 6.2

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gctggtaggccttgagagggcggttaggaagagtggagactgctgcacggactctggaaccATG 65
-----ggtttaggaagagtggagactgctgcacggactctggaaccATG 43

aacatatttgatcgaaagatcaactttgatgcgcttttaaaattttctcatataaccccgtaac 130
aacatatttgatcgaaagatcaactttgatgcgcttttaaaattttctcatataaccccgtaac 108

gcagcagcacctgaagaaggtctatgcaagttttgccctttgtatgtttgtggcggtgcagggg 195
gcagcagcacctgaagaaggtctatgcaagttttgccctttgtatgtttgtggcggtgcagggg 173

cctatgtccatattggtcactcatttcattcaggctggcctgctgtctgccttgggctccctgata 260
cctatgtccatattggtcactcatttcattcaggctggcctgctgtctgccttgggctccctgata 238

ttgatgatttggctgatggcaacacctcatagccatgaaactgaacagaaaagactgggacttct 325
ttgatgatttggctgatggcaacacctcatagccatgaaactgaacagaaaagactgggacttct 303

tgctggatttgcattccttacaggagttggcctgggccctgccctggagttttgtattgctgtca 390
tgctggatttgcattccttacaggagttggcctgggccctgccctggagttttgtattgctgtca 368

accccagcatccttcccactgctttcatgggcacagcaatgatctttacctgcttcaccctcagt 455
accccagcatccttcccactgctttcatgggcacagcaatgatctttacctgcttcaccctcagt 433

NarI
gcactctatgccaggcgccgtagctacctctttctgggaggtatcttgatgtcagccctgagctt 520
gcactctatgccaggcgccgtagctacctctttctgggaggtatcttgatgtcagccctgagctt 498

Leu
gttgcttttgtcttccctggggaatgttttctttggatccatttggCCTttccaggcaaacctgt 585
gttgcttttgtcttccctggggaatgttttctttggatccatttggCCTttccaggcaaacctgt 563

Pro
Phe
atgtgggactggtggtcatgtgtggcttcgtccttTTTgataactcaactcattattgaaaaggcc 650
atgtgggactggtggtcatgtgtggcttcgtccttGTTgataactcaactcattattgaaaaggcc 628

Val
gaacatggagatcaagattatatctggcactgcattgatctcttcttagatttcattactgtctt 715
gaacatggagatcaagattatatctggcactgcattgatctcttcttagatttcattactgtctt 693

SpeI
ACTAGT
cagaaaactcatgatgatcctggccatgaatgaaaaggataagaagaaagagaagaaaTGAagtg 780
cagaaaactcatgatgatcctggccatgaatgaaaaggataagaagaaagagaagaaaTGAagtg 758

accatccagcctttcccaattagacttcctctccttccaccctcatttccttttgcacacatt 845
accatccagcctttcccaattagacttcctctccttccaccctcatttccttttgcacacatt 823

acagggtggtgtgttctgtgataatgaaaagcatcagaaaaaaaaaaaaaaaaaaaa...I.M.A.G.E #207657
acagggtggtgtgttctgtgataatgaaaagcatcagaaaaaaaaaaaaaaaaaaaa.....Genbank #X75861

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I.M.A.G.E. #207657 possesses 22 additional nucleotides in its 5'-UTR that were not present in the original published sequence. and (ii) I.M.A.G.E. #207657 contains two nucleotide substitutions, one at position 568 (C → T), and one at position 621 (G → T). These nucleotide changes result in two amino acid substitutions (P → L and V → F respectively) when compared to the original predicted sequence of Walter *et al.* [293]. The same nucleotide substitutions were also present in the partial hTegt cDNA that I had cloned from granulocyte RNA in Chapter 4 (clone A4AFL8 in Table 4.II, sequence not shown). Therefore, the amino acid substitutions are very likely correct since they were present in 2 independently-derived cDNAs. I have submitted the full sequence of I.M.A.G.E. clone #207657 to GenBank as accession #AF033095.

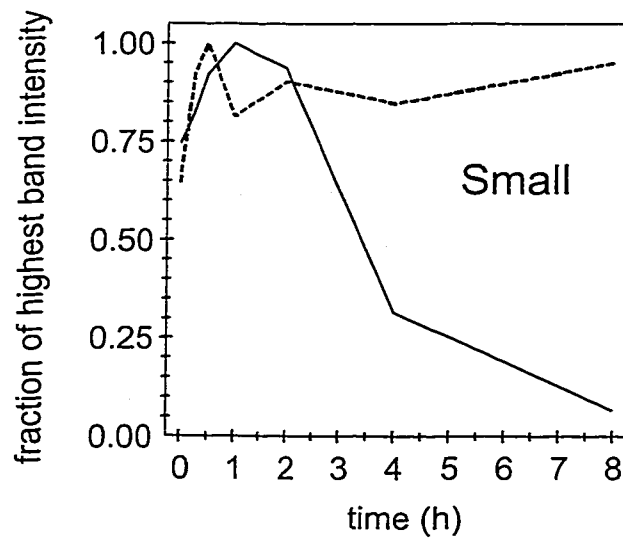
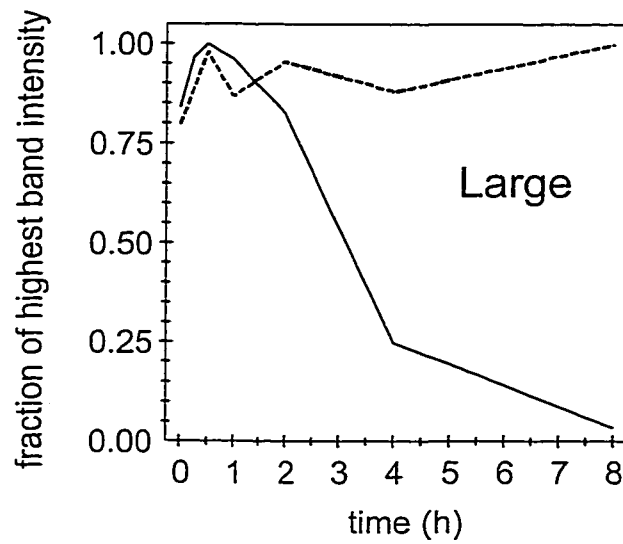
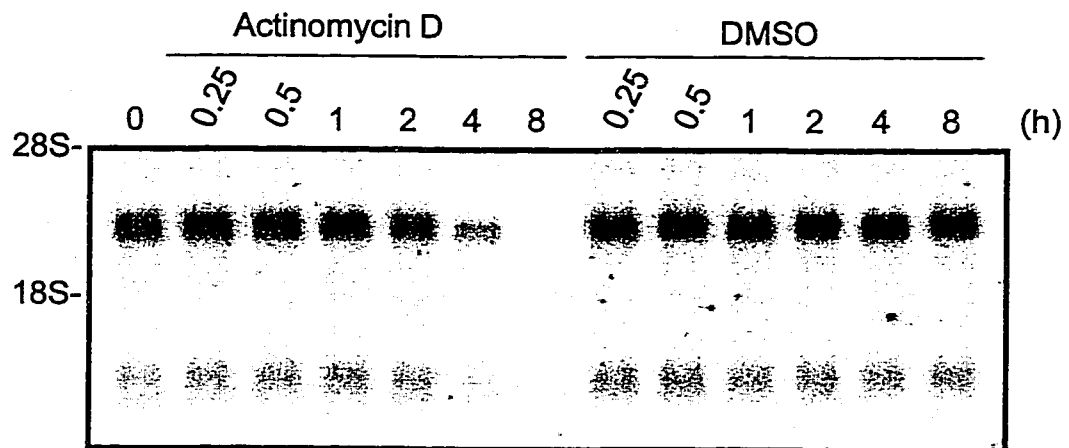
6.4.2 - Determining hTegt mRNA half-life in HL-60 cells

Walter *et al.* found approximately equal amounts of large and small hTegt mRNA in human cells [293]. However, I found that the large transcript was 3-5× more abundant on Northern blots of 4 human cell-types (peripheral blood granulocytes, HL-60, HeLa, and 293T) (e.g., see Figure 6.3 and Chapter 4, Figure 4.5). This difference could not be explained by the probe utilized because its sequence was common to both transcripts. Both transcripts contain the full ORF, but the larger mRNA possesses an additional 1.8 kb of 3'-UTR. This 3'-UTR could enhance mRNA stability, explaining the relative abundance of the large transcript in all cell-types tested.

To assess the effect of the long 3'-UTR on mRNA stability, the half-life of both transcripts was determined by Northern blotting of total RNA from actinomycin D-treated

Figure 6.3. **Half-life determination of large and small hTegt mRNA in HL-60 cells.** 5×10^6 HL-60 cells were cultured in 5 mL DMEM + 10% FCS supplemented with either 4 μ g/mL actinomycin D or 0.1% DMSO as a solvent control. At the indicated times (in h), total RNA was isolated from the cells as described in Chapter 2. One tenth of each sample was subjected to Northern blotting using the ~900 bp *Eco*RI / *Hind*III fragment of I.M.A.G.E. clone #207657 as a probe. The Northern blot is shown in the *top panel*. Using PhosphorImager technology, the bands were quantified and relative band intensities were plotted versus time of treatment. The plot of the large transcript (2.8 kb) is shown in the *middle panel*, while that of the small transcript (1.0 kb) is shown in the *bottom panel*. RNAs isolated from actinomycin D-treated cells are plotted as *solid lines*, while those of DMSO-treated cells are plotted as *dashed lines*.

-141-
Figure 6.3

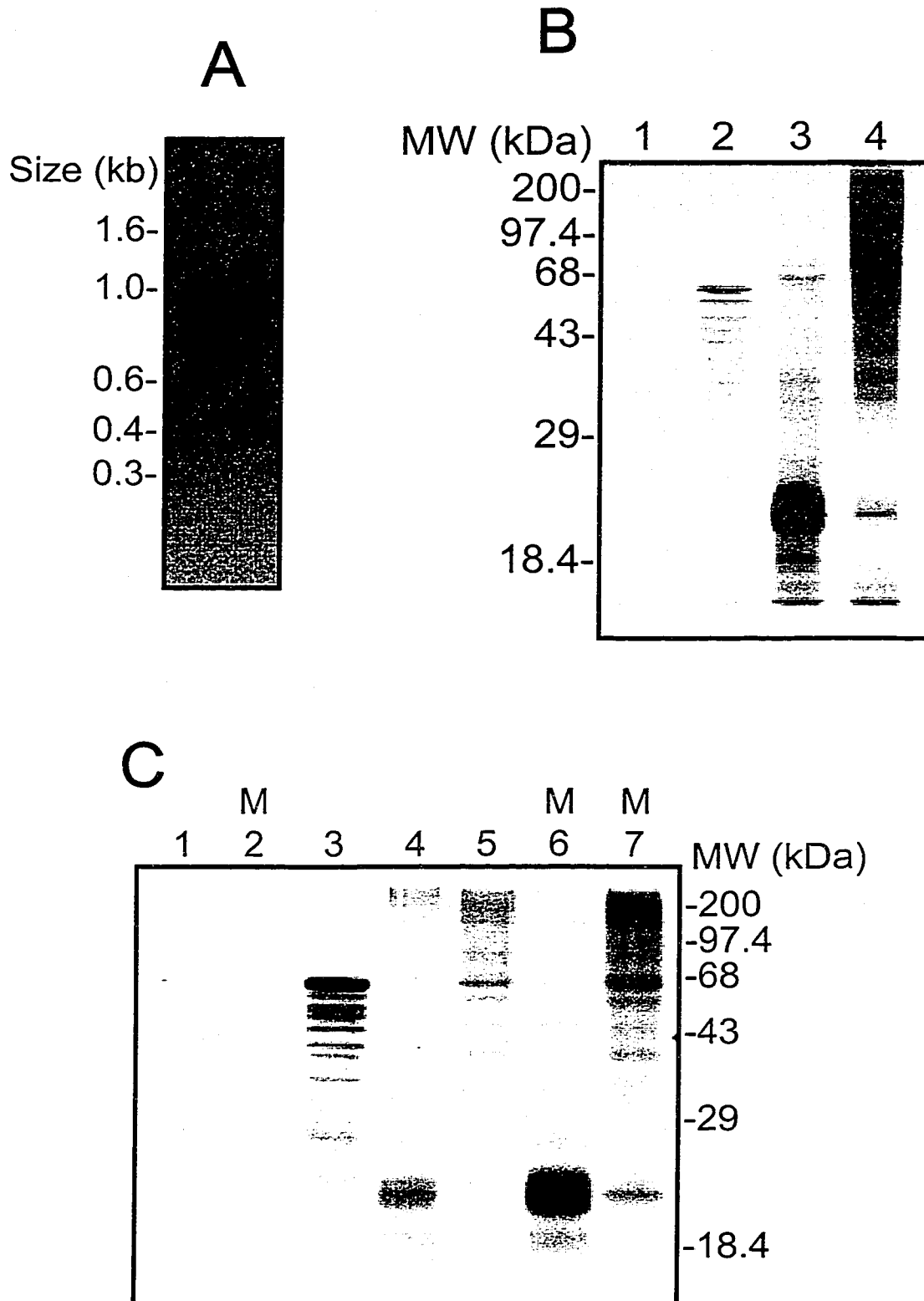


HL-60 cells. The addition of the 3'-UTR had no effect on mRNA stability; both the large and small transcripts had similar half-lives of 3-3.5 h (Figure 6.3). Apparently, the difference in endogenous transcript levels is caused by a polyadenylation site preference, which will be discussed later in this chapter. Therefore, I.M.A.G.E. clone #207657 could be used in transfection studies without modification; the absence of the long 3'-UTR would not be expected to destabilize the transfected mRNA.

6.4.3 - *In vitro* transcription and translation of hTegt

As a first step towards studying the properties of hTegt protein, *in vitro* transcription and translation were performed. T7 RNA polymerase was used to make hTegt mRNA from the I.M.A.G.E. #207657 plasmid. The *in vitro* transcript was electrophoresed on a denaturing agarose gel and stained with ethidium bromide. A single species of the expected size of ~1 kb was observed, verifying its size and integrity (Figure 6.4A). This mRNA was then translated *in vitro* using wheat germ lysates (Figure 6.4B). When the radiolabeled protein was electrophoresed on SDS-PAGE with the standard technique of Laemmli [178], most of the radiolabel ran as a poorly defined smear (Figure 6.4B, lane 4). Laemmli's procedure includes boiling in SDS sample buffer prior to electrophoresis to ensure denaturation. Heat-induced aggregation of hydrophobic plant membrane proteins in the presence of SDS has been observed previously [314]. Since hTegt is predicted to be a very hydrophobic protein also, I tried heating the *in vitro* translated product only to 65°C in SDS sample buffer. Under these conditions, the radiolabeled hTegt electrophoresed consistently as a broad band centred at 22-24 kDa (Figure 6.4B, lane 3). The predicted size of hTegt is

Figure 6.4. ***In vitro* transcription and translation of hTegt.** Procedures were as described in Chapter 2. *Panel A. In vitro* transcription. Capped hTegt RNA was synthesized from I.M.A.G.E. clone #207657 using T7 RNA polymerase. One tenth of the reaction was electrophoresed on a 1.2% agarose gel containing 2.2 M formaldehyde. The ethidium bromide-stained gel is shown with RNA size markers indicated on the left. *Panel B. In vitro* translation using wheat germ lysates. L-[³⁵S]Methionine-radiolabeled protein was synthesized from template RNA with wheat germ lysates following the manufacturer's instructions. *Lane 1*, no RNA added. *Lane 2*, firefly luciferase (supplied by manufacturer). *Lanes 3 and 4*, hTegt. Before electrophoresing on a 12% SDS-PAGE gel, samples were either boiled in SDS sample buffer for 3 min (*lanes 1, 2, and 4*) or were heated in SDS sample buffer for 2 min at 65°C (*lane 3*). *Panel C. In vitro* translation using rabbit reticulocyte lysates. L-[³⁵S]Methionine-radiolabeled protein was synthesized from template RNA with reticulocyte lysates following the manufacturer's instructions. *Lanes 1 and 2*, no RNA added. *Lane 3*, firefly luciferase (supplied by manufacturer). *Lanes 4-7*, hTegt. *M* denotes reactions that were supplemented with canine pancreatic microsomes. Before electrophoresing on a 12% SDS-PAGE gel, samples were either boiled in SDS sample buffer for 3 min (*lanes 1, 2, 3, 5, and 7*) or were heated in SDS sample buffer for 2 min at 65°C (*lanes 4 and 6*). After electrophoresis, the gels depicted in *panels B and C* were stained with Coomassie Blue to visualize molecular weight markers (shown in kDa). Gels were impregnated with En³Hance fluorography agent as per manufacturer's directions, dried and exposed to x-ray film at -80°C to visualize radiolabeled protein.



26.5 kDa. As a positive control, wheat germ lysates were able to synthesize firefly luciferase of 61 kDa (Figure 6.4B, lane 2). Also, no protein was synthesized in the absence of added mRNA, verifying the absence of contaminating endogenous mRNA in the lysates (Figure 6.4B, lane 1).

In vitro translation was next performed using rabbit reticulocyte lysates (Figure 6.4C), since they more closely mimic the translational machinery of the human cell. Similar to that of wheat germ, reticulocyte lysates synthesized hTegt that electrophoresed as a broad band centred at 22-24 kDa (Figure 6.4C, lane 4), and that aggregated when boiled in SDS sample buffer (Figure 6.4C, lane 5). In contrast to wheat germ, reticulocyte lysates translated hTegt poorly unless the reaction was supplemented with canine pancreatic microsomes (Figure 6.4C, compare lanes 4 and 5 to 6 and 7), supporting the notion that hTegt must target to the ER to be translated. Pancreatic microsomes have the capacity to cleave signal peptides [315,316] and to N-glycosylate proteins [317]. However, the electrophoretic mobility of hTegt was similar in wheat germ lysates (with no microsomes) and in reticulocyte lysates supplemented with microsomes (compare Figure 6.4B, lane 3 to Figure 6.4C, lane 6). Therefore, hTegt was not N-glycosylated, nor was a signal peptide removed. Positive and negative controls of reticulocyte lysates were similar to those of wheat germ lysates (Figure 6.4C, lanes 1-3).

6.4.4 - Attempts to raise polyclonal antisera against hTegt

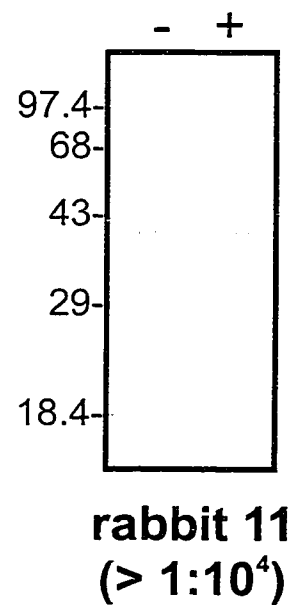
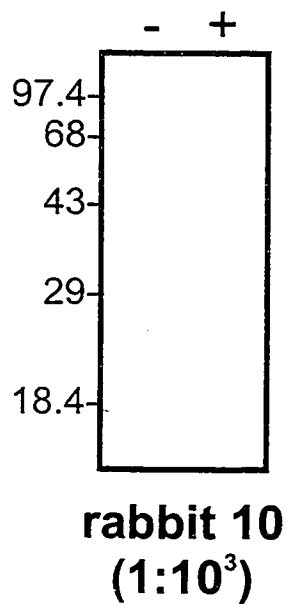
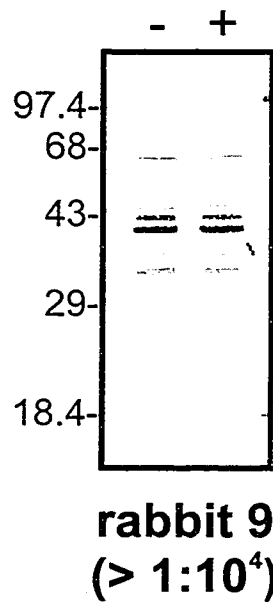
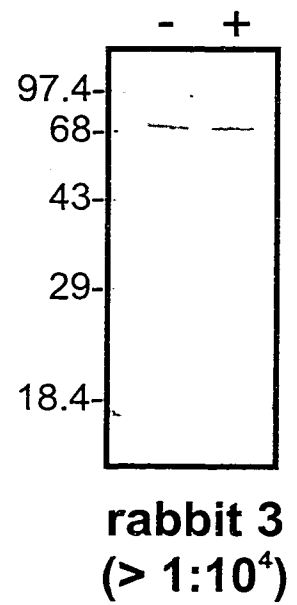
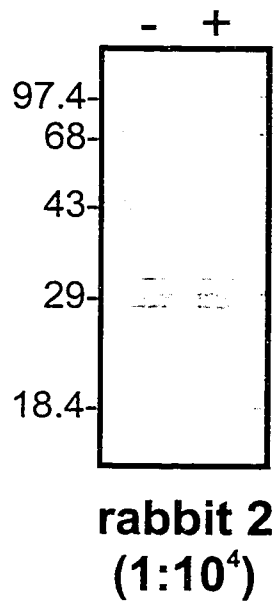
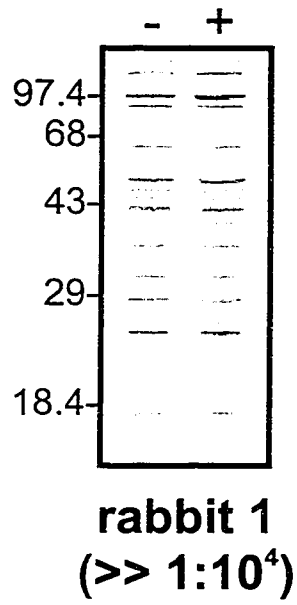
A synthetic peptide corresponding to amino acids 219-233 of the predicted hTegt sequence was made as a tetrameric multiple antigenic peptide (hTegt MAP4) [311]. This

system enhances antigenicity by presenting 4 peptides per molecule linked to a branched polylysine backbone. Six rabbits were injected intramuscularly with the hTegt MAP4. Antisera were collected after the first booster injection and subjected to ELISA using hTegt MAP4 as antigen. Five of the six rabbits demonstrated titres of at least 10^4 , indicating the successful production of antisera. However, none of the antisera could detect *in vitro* translated hTegt by Western blotting (Figure 6.5), nor were any specific bands detected on Western blots of HeLa and 293T extracts (data not shown). Also, only 0-0.5% of *in vitro* translated [35 S]hTegt was immunoprecipitated with each of the six antisera. Therefore, despite obtaining high titres by ELISA, the six antisera were not useful to detect hTegt either by Western blotting or by immunoprecipitation. Potential explanations for this difficulty will be discussed later. The failure to raise antisera against the endogenous hTegt sequence led me to undertake epitope tagging to visualize recombinant protein in transfected cells.

6.4.5 - Toxicity of hTegt overexpression in 293T cells

A monoclonal antibody was commercially available to an immunodominant, 9 amino acid sequence from the influenza virus HA protein (YPYDVPDYA) [318]. This sequence was chosen because it is small and had been used successfully to epitope-tag another integral membrane protein, the rat Na,K-ATPase α 1 subunit [312]. Two HA-tagged hTegt constructs were made, one tagged at the C-terminus (pcDNA3 / hTegt-HA) and one tagged internally within a small hydrophilic region (pcDNA3 / hTe-HA-gt) (see Figure 6.1). Tagging at the N-terminus could have disrupted targeting of hTegt to the ER and was avoided. In addition, an untagged hTegt construct (pcDNA3 / hTegt), and an N-HA-tagged

Figure 6.5. Rabbit polyclonal antisera against the hTegt MAP4 peptide does not detect hTegt by Western blotting. Non-radiolabeled hTegt was synthesized by *in vitro* translation using wheat germ lysates as described in Chapter 2. Both the hTegt *in vitro* translation reaction (+) or a mock reaction containing no RNA (-) were heated at 65°C for 2 min in SDS sample buffer and were electrophoresed on a 12% SDS-PAGE gel. Western blotting was performed as described in Chapter 2. Primary antisera from a total of 6 rabbits was used at a dilution of 1/2000 in TBST. Alkaline phosphatase labeled goat anti-rabbit IgG was used as a secondary antibody at a dilution of 1/2000 in TBST. Molecular weight standards (in kDa) are indicated to the left of each panel. The ELISA titre of the corresponding antiserum (using hTegt MAP4 as antigen) is shown in parentheses under each blot.



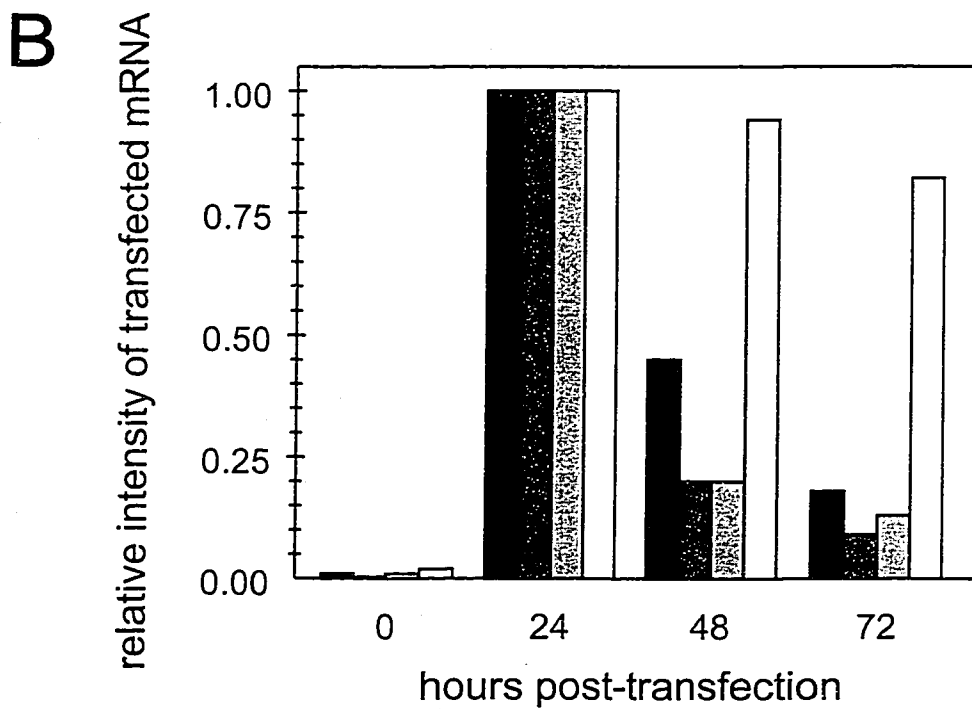
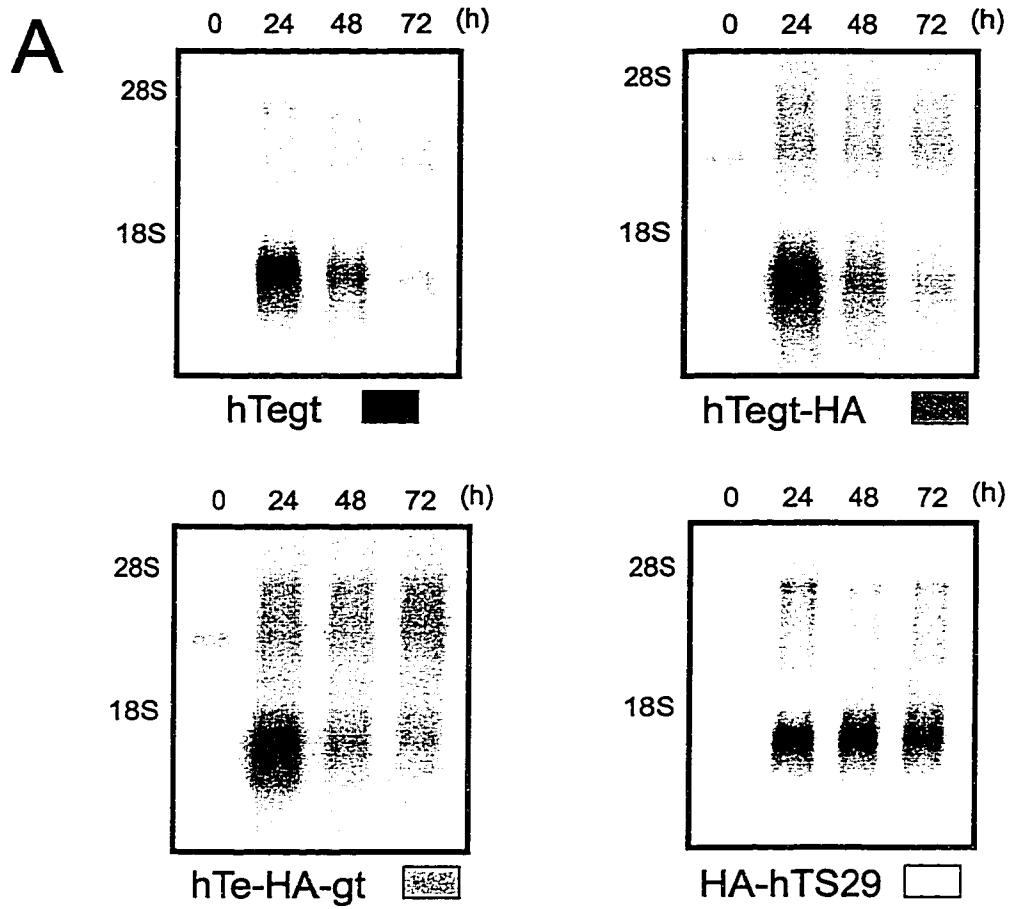
human thymidylate synthase (pcDNA3 / HA-hTS29) were also made as controls (see Figure 6.1). hTS was chosen because it is a well-characterized protein [319-323], and because both a cDNA and anti-hTS polyclonal antibody were available in our laboratory [21].

All constructs contained an SV40 origin of replication in the pcDNA3 backbone (Figure 6.1). 293T cells express SV40 large T antigen which will recognize this origin, replicate the plasmid to high copy number, and overexpress the recombinant protein. To assess the functionality of the 4 constructs, 293T cells were transiently transfected and Northern blotting was performed on total RNA from 0 to 72 h post-transfection (Figure 6.6A). As expected, 293T cells were able to overexpress HA-hTS29 mRNA for at least 72 h with only a slight reduction in band intensity (Figure 6.6B, white bars). However, 293T cells demonstrated a marked loss of overexpressed mRNA from the three hTegt constructs after 24 h (Figure 6.6B, grey and black bars). At 48 h post-transfection of hTegt constructs, significant numbers of cells could be seen floating in the media (data not shown). These cells appeared swollen under phase contrast microscopy and were presumed to be dead. Cell toxicity associated with pcDNA3 / HA-hTS29 transfection was not different from that of vector alone (data not shown). Therefore, overexpression of hTegt and its HA-tagged derivatives was toxic to 293T cells by 48 h. The mode of cell death (e.g., apoptosis) was not investigated.

6.4.6 - Western blotting of transiently transfected 293T cells

Given the results of section 6.4.5, anti-HA immunoblotting was performed on 293T cell extracts at 24 h post-transfection to minimize hTegt toxicity. Protein was extracted

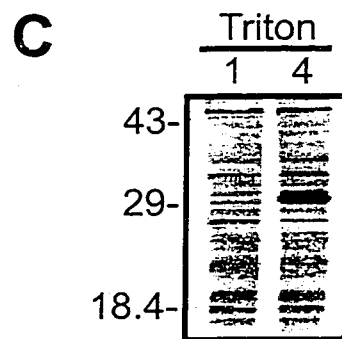
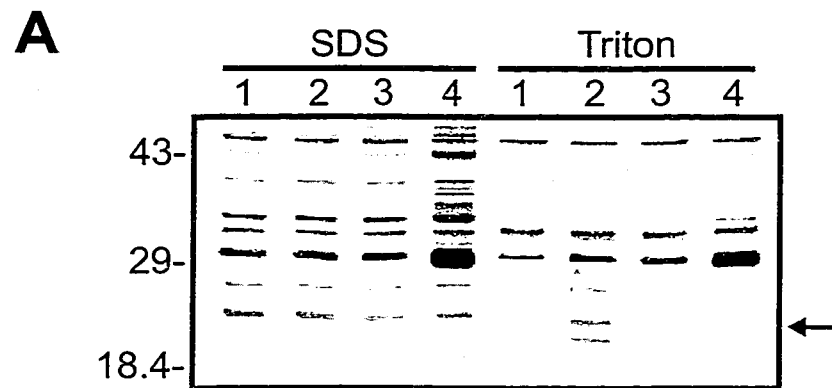
Figure 6.6. hTegt and its HA-tagged derivatives are toxic when overexpressed in 293T cells. 293T cells were transiently transfected with LipofectAmine and the indicated pcDNA3 constructs following the instructions supplied by the liposome manufacturer. Briefly, 80-90% confluent cells in a 6-cm tissue culture plate were transfected with 10-15 μ L LipofectAmine and 4 μ g DNA in serum-free DMEM-HG for 6-7 h at 37°C in a humidified, 5% CO₂ atmosphere. At the indicated times post-transfection (h), total RNA was isolated from the cells. (For the zero time-point, RNA was isolated from cells transfected with pcDNA3 vector alone and allowed to express for 24 h.) 5 μ g total RNA was subjected to Northern blotting as described in Chapter 2. These Northern blots are shown in *part A*. Blots of hTegt and its derivatives were probed with the ~900 bp *Eco*RI / *Hind*III fragment of I.M.A.G.E. clone #207657, while the HA-hTS29 blot was probed with the ~850 bp *Pst*I fragment of pQE-hTS [21]. The bands of overexpressed RNA were quantified by PhosphorImager technology, and were standardized to those of the 24 h time-points. A bar graph of relative signal intensities versus time of expression is shown in *part B*.



either by boiling cells directly in SDS (Figure 6.7A, SDS lanes) or by treating with a nonionic detergent followed by heating to 65°C in SDS (Figure 6.7A, Triton lanes). Recombinant HA-hTS29 of ~29 kDa was detected regardless of the method of protein extraction (Figure 6.7A, lanes 4). hTS has a molecular weight of 36 kDa (Swiss-Prot accession #P04818), but the pcDNA3 / HA-hTS29 construct contained a nonsense mutation derived from *Taq* DNA polymerase (see Figure 6.1). This truncated protein (29 kDa) could be distinguished easily from endogenous hTS (36 kDa) on an anti-hTS immunoblot (Figure 6.7D). Recombinant HA-hTS29 comigrated with a nonspecific band at 24 h post-transfection (compare Figure 6.7A, lanes 1 and 4), but became more prominent if the cells were allowed to express for an additional 24 h (Figure 6.7C).

The electrophoretic mobility of hTegt-HA in Figure 6.7 was similar to that of *in vitro* translated hTegt in Figure 6.4. Ignoring nonspecific signals, hTegt-HA was detected as a weak band at ~23 kDa (highlighted in Figure 6.7 with an arrow) with nonionic detergent extraction only (Figure 6.7A, Triton lane 2). Panel B of Figure 6.7 depicts a darker exposure of panel A to intensify the hTegt-HA band. At least 2 other bands appeared enhanced in the hTegt-HA lanes (Figure 6.7A and B, Triton lane 2). The reason for extra bands is not known, but HA-hTS29 (lanes 4) also showed bands in addition to the main recombinant protein. hTe-HA-gt could not be detected by Western blotting regardless of the method of extraction (Figure 6.7A, lanes 3). The presence of the inframe HA codons was confirmed by DNA sequencing of pcDNA3 / hTe-HA-gt, thus verifying that absence of anti-HA binding was not due to absence of the HA epitope (data not shown).

Figure 6.7. Western blotting of 293T cells transiently expressing HA-tagged pcDNA3 constructs. 293T cells were transiently transfected using LipofectAmine, following instructions supplied by the liposome manufacturer. Briefly, 80-90% confluent cells in a 6-cm tissue culture plate were transfected with 10-15 μ L LipofectAmine and 4 μ g DNA in serum-free DMEM-HG for 6-7 h at 37°C in a humidified, 5% CO₂ atmosphere. Following either 24 h (*panels A and B*) or 48 h (*panels C and D*) of expression time, cell extracts were subjected to Western blotting with either an anti-HA monoclonal antibody (*panels A, B and C*) or an anti-hTS polyclonal antibody (*panel D*) [21]. Extracts were prepared either by boiling cells in SDS sample buffer for 5 min (*SDS lanes*) or by treating cells with 0.5% Triton X-100, 1 mM CDTA, 1 mM PMSF, 2 μ g/mL aprotinin and 20 mM Mops/NaOH (pH 7.2) on ice for 20 min, microcentrifuging for 10 min at 4°C to remove insoluble debris, and then heating supernatants in SDS sample buffer for 2 min at 65°C (*Triton lanes*). Protein extracts were derived from 293T cells that had been transfected with either pcDNA3 alone (*lane 1*), hTegt-HA (*lane 2*), hTe-HA-gt (*lane 3*) or HA-hTS29 (*lane 4*). 10 μ g of total protein was loaded per lane. Protein molecular weight markers are shown to the left of each panel. *Panel B* shows a darker exposure of *panel A* to intensify the hTegt-HA band that is highlighted with an *arrow*.



6.4.7 - Immunofluorescence of transiently transfected HeLa cells

Subcellular localization of hTegt by Western blotting of subcellular fractions was not attempted because of the prior difficulties encountered with hTegt on SDS-PAGE. It was decided that immunofluorescence of fixed cells was the better option. HeLa cells were transiently transfected with various pcDNA3 constructs and anti-HA epifluorescence microscopy performed. HeLa cells do not express SV40 large T antigen and will not overexpress transfected protein. No obvious hTegt toxicity was observed with these cells up to 72 h post-transfection. Similar to that seen with Western blotting, anti-HA antibody could detect only HA-hTS29 and hTegt-HA proteins; no significant signal could be seen when HeLa expressed hTe-HA-gt (Figure 6.8). As expected, HA-hTS29 showed a cytoplasmic and nuclear localization (Figure 6.8). Considering that hTegt is likely an integral membrane protein and that it targets to the ER during translation, four subcellular localizations are possible (ER, Golgi, lysosomes/other vesicles, and plasma membrane). Comparing the anti-HA immunofluorescence signal of hTegt-HA in Figure 6.8 with the DIC image of the same field, the protein was likely found in the ER, although some Golgi staining cannot be ruled out. In addition to the ER signal, some cells also demonstrated clear staining surrounding the nucleus (data not shown). The localization was verified by using higher resolution anti-HA confocal immunofluorescence microscopy. In the Z-axis slices shown in Figure 6.9, a strong anti-HA signal can be seen as a thick, discontinuous rim around the nucleus. Therefore, based on both confocal and epifluorescence, hTegt-HA localized mainly to the ER and contiguous structures (i.e., the nuclear membrane).

Figure 6.8. Immunofluorescence of HA-tagged proteins transiently expressed in HeLa cells. HeLa cells were plated on sterile coverslips, transfected with various pcDNA3 constructs, and epifluorescence microscopy carried out as described in section 6.3.6. For the four different constructs used, representative pictures are shown of the anti-HA immunofluorescence and the same field under DIC. All fields are shown at 400× magnification.

-152-
Figure 6.8

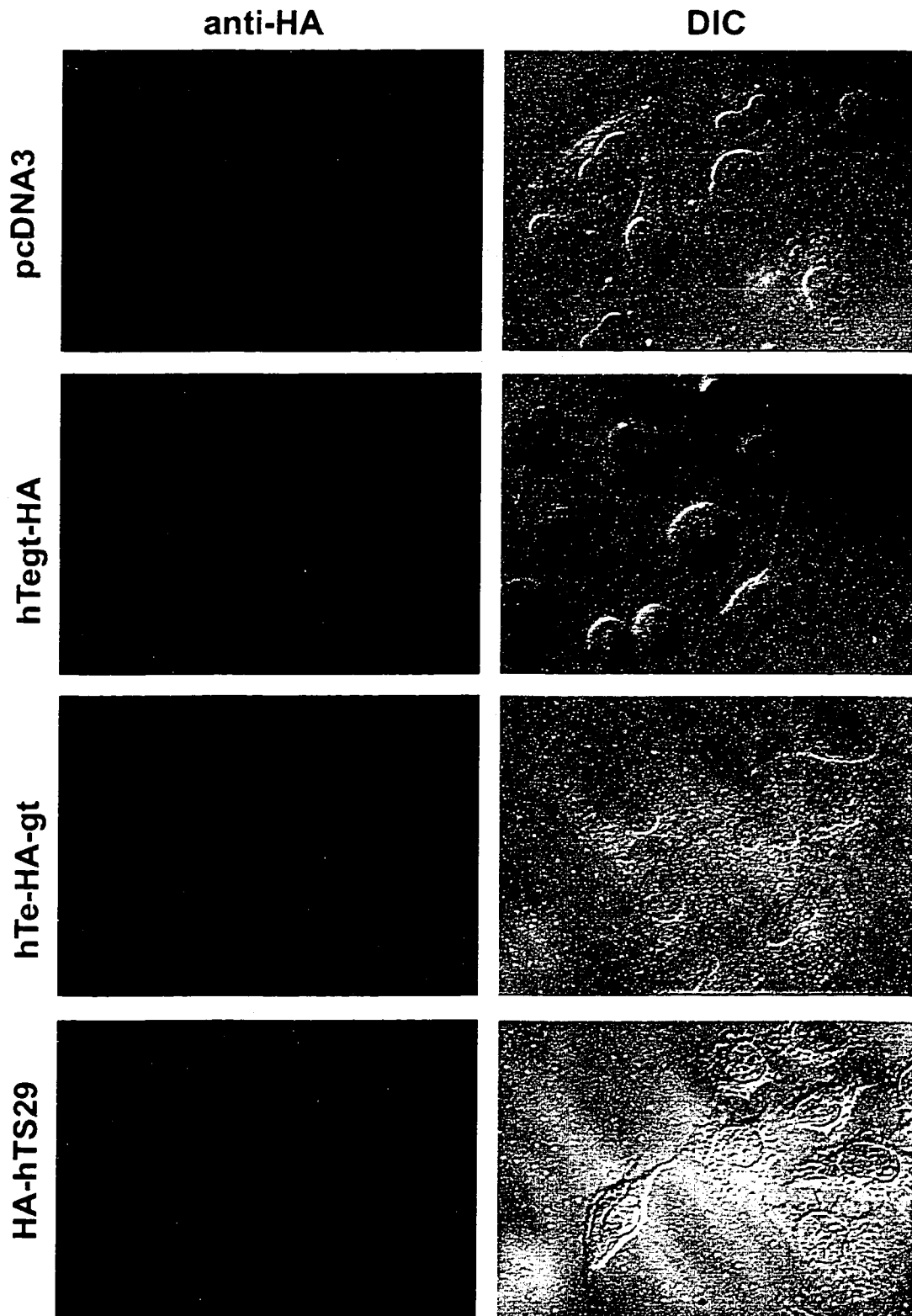
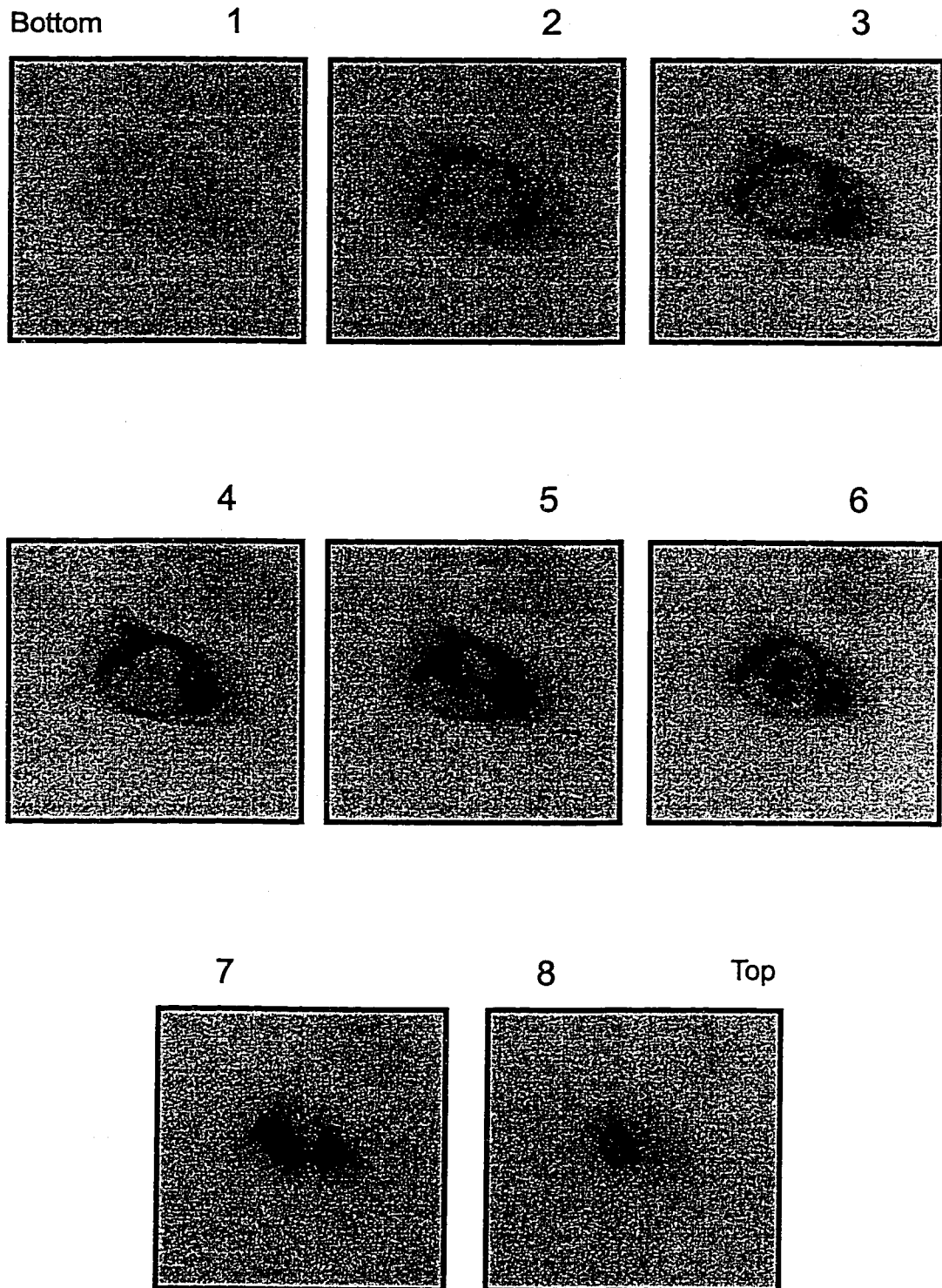


Figure 6.9. Confocal immunofluorescence microscopy of a HeLa cell transiently expressing hTegt-HA. HeLa cells were plated on sterile coverslips, transfected with pcDNA3 / hTegt-HA, and immunofluorescence carried out as described in section 6.3.6. Eight confocal images were acquired at 2 μm intervals along the Z-axis from the bottom of the cell (image #1) to the top (image #8). The images are shown in inverted grayscale to improve contrast. Magnification $\sim 1000\times$.



6.5 - Discussion

This chapter details the study of the human testis-enhanced gene transcript (hTegt) protein, with the ultimate goal of determining its function in mammalian cells. Although ubiquitously expressed and conserved between human and rat, the protein contains no consensus sequence that would hint at its intracellular role. Despite considerable analysis described in this chapter, the function of the protein remains elusive. As with some hydrophobic proteins, the study of hTegt has proven difficult. However, knowledge of its properties that has been gained throughout this study may be useful for future experiments. hTegt mRNA is expressed at relatively high levels in many cell-types including mature human neutrophils. Whatever its function, its ubiquitous presence suggests a protein with a vital, housekeeping role. Continued study is warranted.

The sequence of the ORF of hTegt originally submitted to GenBank by Walter *et al.* (accession #X75861) was verified by sequencing I.M.A.G.E. clone #207657, with the exception of 2 nucleotides. The resulting amino acid substitutions (Leu¹⁶⁹ and Phe¹⁸⁷) make the human Tegt sequence identical to that of the rat at these positions, rather than different as was originally believed. Considering that Tegt is highly conserved, it seems unlikely that this represents a polymorphism in the human population. Missense mutations incurred during cloning or sequencing errors in #X75861 are the more likely explanations. Our sequence is reliable since it was verified in two independently-derived cDNA clones.

Two mRNAs of different sizes are transcribed from the Tegt gene due to the use of different polyadenylation sites. Examples of other gene products known to be differentially polyadenylated are numerous and include murine Surf-4 [324], human Bcl-2 [325,326],

human fatty aldehyde dehydrogenase [327] and human Timp3 [328]. All studies of hTegt protein in this chapter have utilized the smaller of the 2 transcripts, although the same protein should be translated from the larger mRNA. Despite having similar half-lives, the ratio of large to small transcript in HL-60 cells is 5:1. Small and large hTegt are generated by two uncommon polyadenylation signals, AATGAA (nucleotides 839 to 844 of GenBank accession #X75861) and AATGAC (nucleotides 2549 to 2553 of GenBank accession #X75861) respectively [293]. Apparently, the polyadenylation machinery of HL-60 cells prefers the second of these uncommon sites; this second site is chosen $5/6 \approx 85\%$ of the time. The reason for this preference is not known, but could be caused by nucleotide sequences in or near the polyadenylation site itself. A reasonable assumption is that "second polyadenylation site preference" also occurs in other human cell-types.

Based on similar electrophoretic mobilities of *in vitro* translated hTegt protein and transiently overexpressed hTegt-HA protein from 293T cells, no appreciable N-glycosylation, O-glycosylation or proteolytic processing occurred. hTegt-HA was localized mainly to the ER and nuclear membrane by immunofluorescence. O-glycosylation of ER resident proteins is minimal or absent since O-linked carbohydrates are added sequentially as monomers during passage through the Golgi [329]. I cannot eliminate the possibility that O-oligosaccharides were present, but they would have to be small, immature and have little effect on SDS-PAGE mobility. Since the sequence of hTegt is known, minor O-glycosylation could be detected by molecular mass estimation of Edman degradation products. However, if present, O-glycosylation is likely unnecessary for proper functioning of the protein. Also, both the putative N-glycosylation site and bipartite NLS predicted by

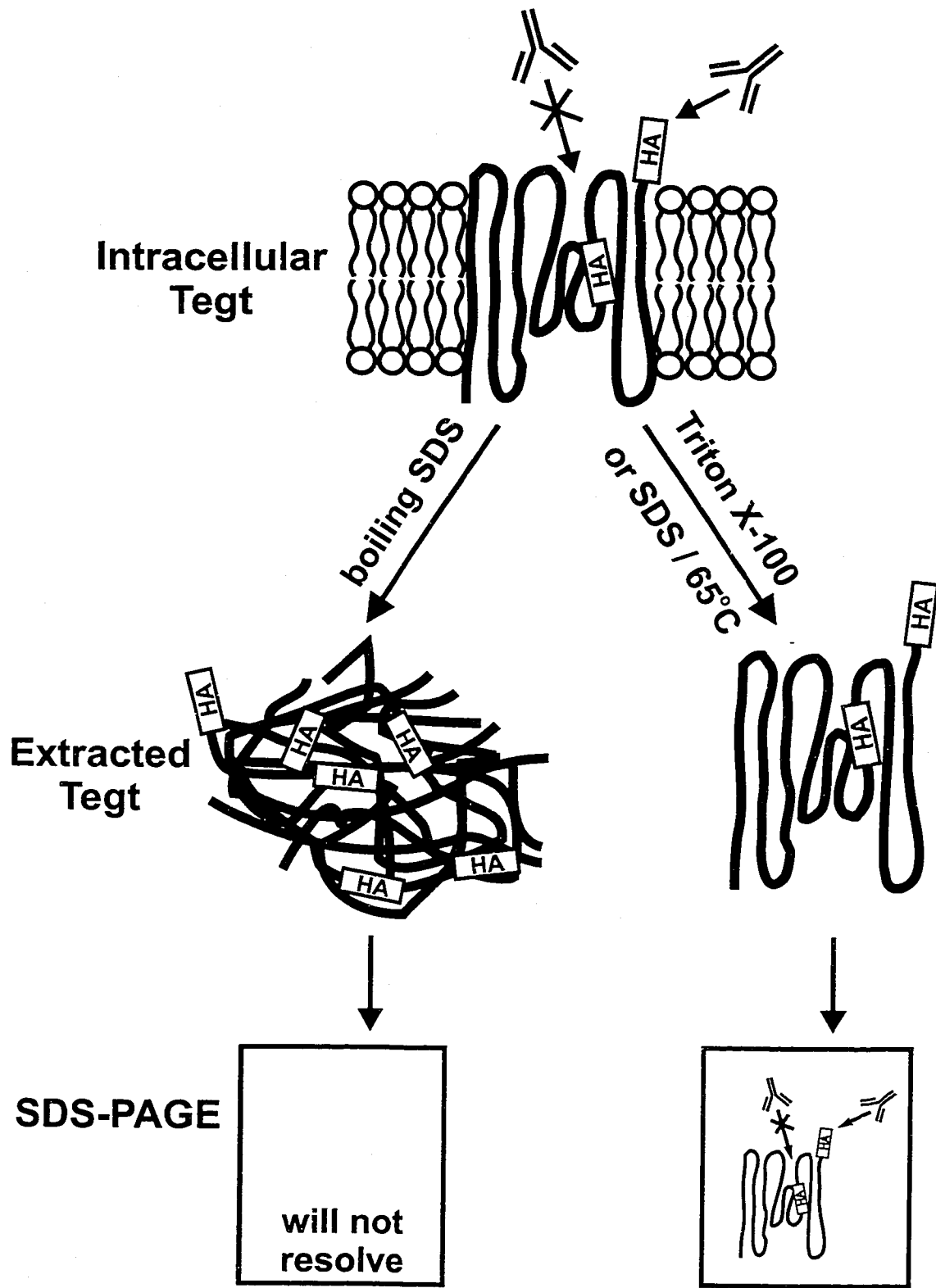
Walter *et al.* [293] are apparently nonfunctional.

When heated to 65°C in SDS, both *in vitro* translated hTegt and transiently overexpressed hTegt-HA electrophorese at an apparent mw of ~23 kDa, and not at the predicted molecular weight of 26.5 kDa. When electrophoresed on a highly cross-linked SDS-PAGE gel, *in vitro* translated hTegt migrated at a higher apparent mw than the 26-27 kDa p27gran protein (Chapter 5, Figure 5.6B, lanes 1 and 2). A likely explanation is that hTegt does not fully denature at 65°C in SDS, and retains partial secondary and tertiary structure. Sieving of the protein through the pores of a polyacrylamide gel is affected by this nonlinear structure, similar to that of supercoiled plasmid DNA on agarose gels. Thus, nondenatured hTegt migrates faster than expected on loosely cross-linked PAGE, but slower than expected on tightly cross-linked PAGE. Boiling hTegt in SDS aggregates the protein with itself (homotypically) and/or with other proteins (heterotypically). An explanation is that addition of sufficient energy in the form of heat causes hTegt to denature, but SDS cannot maintain the protein in a monomeric state. The same hydrophobic interactions that prevent the protein from denaturing at 65°C, cause the protein to aggregate after boiling. Similar heat-induced aggregation of a protein in SDS has been observed with presenilin 1 [330], the H⁺/monoamine antiporter [331], the 27 kDa component of ammonia monooxygenase from *Nitrosomonas europaea* [332], and numerous uncharacterized membrane proteins from corn roots [314,333]. These are all highly hydrophobic integral membrane proteins, and it is believed that the aggregation is a result of this hydrophobicity [332]. In the case of the H⁺/monoamine antiporter, the aggregates were so stable that only anhydrous trifluoroacetic acid could dissociate them [331].

The HA-epitope in hTegt-HA is exposed at the C-terminus and available to bind antibody in fixed cells. The epitope remains exposed on SDS-PAGE when the protein is heated to 65°C. However, the HA-epitope in hTe-HA-gt is masked within secondary and tertiary structure and cannot bind antibody in fixed cells. This internal HA-epitope is still hidden on SDS-PAGE if the extract is heated to 65°C because the protein is not denatured. Although the internal HA-tag was placed in a predicted hydrophilic loop, apparently this amino acid sequence is not exposed sufficiently to bind anti-HA antibody. Boiling the tagged proteins in SDS causes them to aggregate, and neither can be resolved on SDS-PAGE. This hypothesis is depicted diagrammatically in Figure 6.10. The model also explains the inability of hTegt MAP4 antisera (see results section 6.4.4) to detect hTegt by immunoprecipitation or by Western blots, despite possessing high ELISA titre against the synthetic peptide. The first 9 of the 15 amino acids of hTegt MAP4 are within the last predicted transmembrane region (Swiss-Prot accession #P55061) which could explain antibody inaccessibility. To solve the problem of epitope masking, hTegt must remain a denatured monomer during gel electrophoresis. A technique using phenol-acetic acid-urea polyacrylamide gel electrophoresis (PAU-PAGE) was developed to resolve problematic plant membrane proteins [314]. This procedure could work, but is technically difficult and may not be compatible with Western blotting. Another possibility would be to replace SDS with another sulfated hydrocarbon that possesses a longer carbon chain and thus greater hydrophobicity. Such systems need testing, but if successful would greatly facilitate any future studies of Tegt.

TS is a cytoplasmic and nuclear protein, essential for dTMP *de novo* synthesis and

Figure 6.10. Hypothesized effect of hTegt conformation on anti-HA antibody binding both *in situ* and on Western blots.



DNA replication [322,323]. HA-hTS29 demonstrated both cytoplasmic and nuclear staining in transiently transfected HeLa cells. HA-hTS29 expression was not toxic by 72 h in both HeLa and 293T cells despite a truncation at the C-terminus that could prevent enzymatic activity. Although not toxic when expressed at lower levels for 72 h in HeLa cells, hTegt expression at higher levels in 293T cells was toxic after 24 h. Thus, it appears that overexpression cannot be tolerated despite the presence of high mRNA levels in most cell-types. The reason for the toxicity and the mode of cell death have not been studied. Toxicity resulting from overexpression is a phenotype that might be useful in future studies of the function of Tegt. For example, if Tegt is an integral membrane ion/molecule channel, the ability of various inhibitors of ion transporters to abrogate hTegt toxicity could be tested.

hTegt-HA localized to the ER and nuclear membrane by anti-HA immunofluorescence of transfected HeLa cells. The possibility that the HA epitope prevented proper localization of the protein cannot be discounted entirely. Although greater confidence could be obtained by tagging the protein in different positions, tagging is not straightforward because Tegt contains few hydrophilic regions. Even tagging within a hydrophilic region did not guarantee antibody access, as was demonstrated with hTe-HA-gt. Raising antisera against the endogenous sequence using mixed synthetic peptides would seem to be the best choice. Toward the goal of elucidating the function of Tegt, phenotypic analysis of cells after treatment with antisense oligonucleotides could prove useful. However, complete removal of sense mRNA would be difficult because hTegt is an abundant message. Now that the entire genome of *Saccharomyces cerevisiae* is known, low stringency searching of GenBank has revealed a putative yeast homolog of Tegt. This putative protein

(ORF YNL305c, GenBank accession #1302403) possesses ~25% amino acid identity to hTegt. Producing a yeast Tegt knockout is another possible approach to elucidating a function for this enigmatic protein.

Chapter 7

General Conclusion

Several novel aspects of neutrophil transcription and translation have been identified in the work of this thesis. GM-CSF was shown to increase *hsgk* mRNA ~10-fold and an unknown 26-27 kDa protein (p27gran) ~3-fold. The sequence of p27gran remains unknown despite several attempts to determine it. The positive identification of this protein would be of interest since it demonstrated the largest increase in translation with GM-CSF. My identification of *hsgk* is the first reported in leukocytes and is the first indication of its regulation by a cytokine. Expression of this putative serine/threonine kinase is not restricted to leukocytes; *sgk* can be induced transcriptionally with various stimuli in a broad range of cell-types. However, its function and intracellular substrates are currently not known. The expression of *sgk* has been correlated with cell cycle progression as well as ion imbalances induced by hypertonic conditions. Since neutrophils do not undergo cell division, the former correlate seems to be of little significance. Given the connection between apoptosis and cell shrinkage/intracellular acidification, the latter correlate could be of importance in the apoptotic delay induced by GM-CSF.

GM-CSF was found to have no effect on the expression of Bcl-2 family members. Of the anti-apoptotic Bcl-2, Mcl-1 and Bcl-X_L proteins and the pro-apoptotic Bax protein, only Bax was found to be expressed at detectable levels. The Bcl-2 family continues to grow (at least 3 more were identified in the course of writing this thesis, namely the pro-apoptotic Bok and Bim [334,335] and the anti-apoptotic Bcl-w [336]). Although not all family members have been analysed in human neutrophils, it is becoming increasingly apparent that Bcl-2 and its homologues are not involved in the cytokine-induced apoptotic delay. Other mechanisms must be invoked.

GM-CSF-stimulation of neutrophils can increase [³H]uridine incorporation by as much as 10-fold in short-term cultures of ≤ 6 h. At least 90% of the radiolabeled RNA is derived from RNA polymerase II, but most of it is nonpolyadenylated and found in the nucleus. Neutrophils appear to be sluggish in synthesizing mature cytoplasmic mRNA. Northern blotting experiments using *hsgly* intron probes have implicated transcriptional elongation as the rate limiting step for this transcript. Analysis of other transcripts is required to gain greater confidence. However, it is intriguing to hypothesize that sluggish mRNA synthesis either brought about the loss of subcellular structures in the differentiating neutrophil or that the loss of subcellular structures (particularly the ribosome) has slowed transcription by limiting the amount of some critical protein(s). I favour the latter hypothesis, although there is no direct evidence currently to support such an opinion.

Finally, the ubiquitously expressed, highly conserved protein, hTegt, was studied. The function of this membrane protein still remains obscure, but several of its properties determined in chapter 6 could be helpful in future work. Although the hTegt mRNA is strongly expressed in human neutrophils, GM-CSF does not effect its expression. hTegt appears to be a housekeeping gene, possibly a membrane channel located in the ER. Considering the scarcity of ER and the low level of synthesis of secreted protein in mature human granulocytes, questions arise regarding the significance of hTegt in these cells. Once a function has been ascribed to the protein, its purpose in granulocytes should become clearer. Many questions still need to be addressed.

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Appendix I

Identification of contaminating *Pseudomonas fluorescens* in human granulocyte preparations by the presence of [³²P]polyphosphate.

AI.1 - Summary

[^{32}P]polyphosphate ([^{32}P]poly(P)) was identified as a significant component in $^{32}\text{P}_i$ -radiolabeled human granulocytes, ranging from 10-76% of the total incorporated radiolabel. The identity of the [^{32}P]poly(P) was verified by its characteristic (i) precipitation with Ba^{2+} , (ii) lability to acid-catalyzed hydrolysis, (iii) partial alkaline hydrolysis to form a 'phosphate ladder', (iv) conversion to [^{32}P]trimetaphosphate, (v) hydrolysis to $^{32}\text{P}_i$ by yeast exopolyphosphatase, and (vi) lability to divalent cation-catalyzed hydrolysis above pH 7. After further analysis, the poly(P) signal was found to originate from a low-level contamination of *P. fluorescens*, present in some of the solutions used to isolate and radiolabel the granulocytes. The solutions were originally filter-sterilized but were not kept sterile thereafter. These bacteria were overlooked initially using routine microscopy because 1 bacterium per 100 granulocytes was estimated to be present. I conclude that low-level microbial contamination can be a significant problem when studying poly(P) by radiolabeling intact mammalian cells or subcellular fractions with $^{32}\text{P}_i$, techniques that have been used previously. A preliminary search for [^{32}P]poly(P) in intact human fibroblasts radiolabeled with $^{32}\text{P}_i$ for 24 h revealed a low poly(P) content, ~ 1 pmole phosphate per 10^6 cells. However, the presence of significant amounts of other contaminating radiolabeled species in the fibroblasts made this quantitation unreliable.

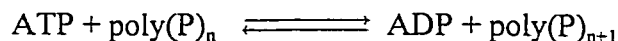
AI.2 - Introduction

Inorganic polyphosphates (poly(P)) are linear polymers of condensed P_i linked by phosphoanhydride bonds. The length of poly(P) varies, but can exceed 1000 phosphate

residues [1,2]. Figure AI.1 illustrates the structure of linear poly(P) as well as 2 other known classes. The presence of poly(P) has been reported in a wide range of organisms including bacteria, fungi, algae, mosses, insects, protozoa, and in the tissues of higher plants and animals [2-5]. These polymers have been studied most extensively in microorganisms, where poly(P) can accumulate in large quantities. By contrast, relatively few studies of higher eukaryotes have been reported, but when found, the amounts are very low compared to those in microorganisms. For example, yeast can accumulate poly(P) to 20% of their dry weight when cultured after phosphate starvation [2], while poly(P) in rodent tissues was recently quantified at 25 to 120 μM P_i residues [5]. In rat liver nuclei and human fibroblast lysosomes, the presence of poly(P) has been reported using $^{32}\text{P}_i$ -radiolabeling [6-8], but its function in these organelles remains to be established.

Intracellular levels of poly(P) are controlled by both anabolic and catabolic reactions. Biosynthetic and hydrolytic enzymes have been identified. Four examples are listed below.

- (i) Polyphosphate: ADP phosphotransferase (EC 2.7.4.1) (also called poly(P) kinase)



- (ii) Polyphosphate: D-glucose 6-phosphate phosphotransferase (EC 2.7.1.63) (also called poly(P) glucokinase)

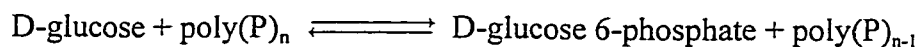
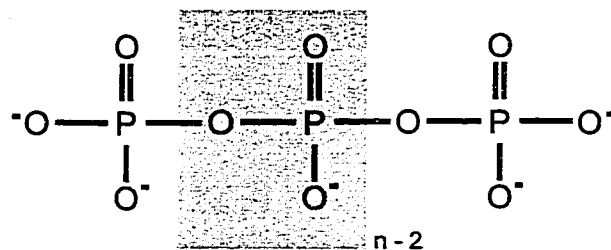
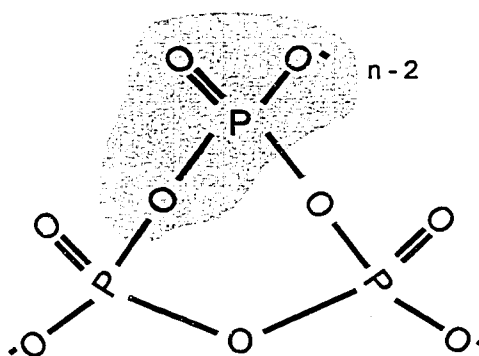


Figure AI.1. **Three classes of condensed inorganic phosphates.** X can be any monovalent cation. Ultraphosphates are of no biological significance since the branch-points are easily hydrolyzed in aqueous solution. The abbreviation poly(P) used in this appendix refers to linear polyphosphate. [3]

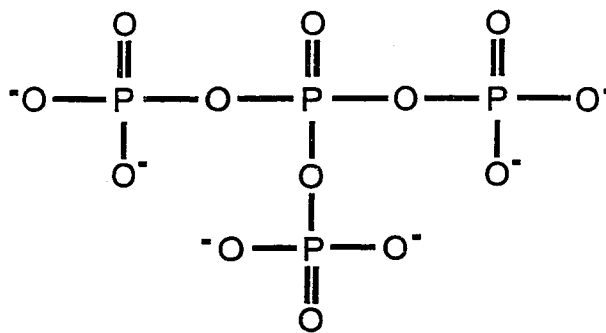
A) Linear Polyphosphate ($P_nO_{(3n+1)}X_{n+2}$)



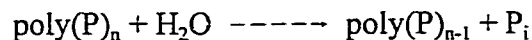
B) Cyclic Metaphosphate ($P_nO_{3n}X_n$)



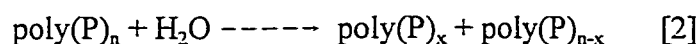
C) Branched Ultraphosphate ($P_4O_{13}X_6$)



(iii) Exopolyphosphatase (EC 3.6.1.11)



(iv) Endopolyphosphatase (EC 3.6.1.10)



Examples of all four enzymes have been found in many microorganisms. *E. coli* is known to contain both poly(P) kinase and exopolyphosphatase activities [9,10]. Exopolyphosphatase and endopolyphosphatase have been purified to homogeneity from *S. cerevisiae* [11,12]. The poly(P) glucokinase from *Mycobacterium tuberculosis* has been actively studied; this enzyme also possesses classical glucokinase activity, utilizing ATP as a phosphate donor [13]. Endopolyphosphatase activity has been partially purified from bovine and rat brain [12], but no anabolic enzyme has been isolated from mammalian systems to date.

In microorganisms, postulated functions of poly(P) include (i) precise regulation of intracellular P_i levels [2], (ii) donation of 'high-energy' phosphates in kinase reactions [14,15], (iii) intracellular sequestering of divalent cations [2], (iv) buffering of alkaline stress by rapid hydrolysis to tripolyphosphate [16], and (v) facilitation of DNA, Ca^{2+} and P_i transport across membranes [17,18]. Although an arguable generalization, these functions pertain to the use of poly(P) as archaic phosphagens or as protection against extreme

environmental stresses. The latter possibility was supported by the recent development of *E. coli* poly(P) kinase null mutants [19]. Poly(P) kinase is responsible for the synthesis of high molecular weight poly(P) in *E. coli*. Although displaying no gross phenotypic changes, the mutants showed increased sensitivity to heat shock, H₂O₂, and prolonged nutrient deprivation. Taking into consideration the postulated functions of microbial poly(P), there is no apparent reason for the presence of low quantities in mammalian cells. Despite reports of its identification, the function of the polymer in mammalian cells remains obscure.

AI.3 - Materials and Methods

AI.3.1 - Purification of [³²P]poly(P)

Radiolabeled cells were washed and sonicated extensively in 200 µL of 1 M urea, 0.2% (w/v) SDS, 5 mM CDTA, 1 mM sodium phosphate and 10 mM Mops/NaOH (pH 7.5) (10-20 s at level 2 using a 50 W Microson XL2005 fitted with a 1/8" microprobe). Proteinase K was added to a concentration of 0.5 mg/mL and samples were digested at 37°C for 16 h. Samples were then extracted with 1 vol. phenol/CHCl₃ (1 g phenol per mL chloroform). After removal of the aqueous phase, the residue was back-extracted with ½ vol. sonication buffer lacking SDS and urea. The pooled aqueous phase was extracted once with CHCl₃. Sodium phosphate glass (50-250 µg of type 35) was added as a carrier [8]. Poly(P) was isolated at room temperature using a modification of an earlier procedure [20] as follows. The mixture was brought to ~1% barium acetate using a stock of 50% (w/v) barium acetate/HCl (pH 4.5). The precipitate that formed was collected by centrifugation at 13,000 × g. The supernatant was discarded and the pellet washed with water. Ba²⁺ was removed by

adding ~100 μL of a 50% suspension of Spectra/Gel cation exchange resin 50X8 (sodium form in 20 mM Mops/NaOH (pH 7.5)). The soluble phase was recovered and the resin washed with water to remove any trapped radioactivity. The combined liquid phases were concentrated to an appropriate volume under vacuum.

AI.3.2 - Acid molybdate assay for $^{32}\text{P}_i$

The $^{32}\text{P}_i$ content of samples was determined using a modified acid molybdate extraction [21]. Samples were diluted to 300 μL containing 50 nmole of sodium phosphate as a carrier. 150 μL of 2.5% (w/v) ammonium molybdate in 1.2 N HCl was added, and the phosphomolybdic acid that formed was extracted into 1-hexanol ($3 \times 200 \mu\text{L}$). The radioactivity present in both the aqueous (non- $^{32}\text{P}_i$) and hexanol ($^{32}\text{P}_i$) phases was determined by Cerenkov counting [22].

AI.3.3 - Electrophoretic analysis of [^{32}P]poly(P)

AI.3.3.1 - 20% gels.

Electrophoretic analysis was carried out using a $38 \times 50 \times 0.04$ cm DNA sequencing gel (Bio Rad). Samples were diluted in electrophoresis buffer (1 mM CDTA, 20 mM sodium acetate and 40 mM Tris/acetic acid (pH 7.8)) and brought to 5% (w/v) sucrose, 0.005% (w/v) bromophenol blue. Polyacrylamide gels were 20:5 (%T:%C) acrylamide in electrophoresis buffer with 7 M urea. Gels were pre-run for 60 min at 1500 V, then samples were loaded and run for 2¼ h at approximately 1650 V (maximum 125 W). Gels were transferred to aluminum foil and subsequently dried onto Whatman 3MM chromatography paper using a

gel dryer [23]. Dried gels were exposed to preflashed x-ray film at -100°C .

AI.3.3.2 - 12% gels.

Electrophoresis was performed using the Mini-PROTEAN II system (Bio Rad) with $80 \times 73 \times 0.75$ mm polyacrylamide gels containing 12:5 (%T:%C) acrylamide and electrophoresis buffer. Samples were diluted with electrophoresis buffer containing sucrose and bromophenol blue as above. $5.0 \mu\text{L}$ of each sample was loaded per lane, and the gel electrophoresed at 200 V until the tracking dye had migrated $\sim 2/3$ down the gel. The gels were dried and exposed to film as in section AI.3.3.1.

AI.3.4 - Preparation of synthetic $[^{32}\text{P}]\text{poly}(\text{P})$

Ten mg monobasic sodium phosphate and $[^{32}\text{P}]\text{orthophosphate}$ were combined at the tip of a conical Pyrex centrifuge tube, loosely plugged with glass wool. The mixture was gently heated with a Bunsen burner flame until all liquid had evaporated, then strongly heated over a 5 to 10 min period. Strong heating was carried out intermittently at a temperature which caused the emission of orange (sodium) light from the Pyrex tube. The resulting 'phosphate glass' was dissolved in 50 mM NaOH/10 mM CDTA. Any insolubles were removed by centrifugation before sampling.

AI.3.5 - Preparation and analysis of $[^{32}\text{P}]\text{trimetaphosphate}$

The $[^{32}\text{P}]\text{poly}(\text{P})$ sample was evaporated to dryness under vacuum, then dissolved in $50 \mu\text{L}$ TMP_i buffer (0.6 mM MgCl_2 and 100 mM Tris/HCl (pH 8.0)). $[^{32}\text{P}]\text{trimetaphosphate}$ was not detected in the extracts at this point. The solution was overlaid with silicone oil

and heated at 70°C for 70 h [11]. After addition of CDTA to 10 mM to chelate Mg^{2+} , 1 μ L of the aqueous phase was spotted onto a prewashed PEI-cellulose TLC sheet and chromatographed with freshly prepared 0.3 M NH_4HCO_3 , 2 mM CDTA [24]. [^{32}P]trimetaphosphate was visualized by autoradiography, cut from the sheet, and quantified by liquid scintillation counting. The position of trimetaphosphate ($R_f \approx 0.45$) was verified by running a commercial preparation on the same chromatogram. The latter was visualized by spraying with 2 N HCl, heating at 80°C for 15 min, and then spraying with 4.2% (w/v) ammonium molybdate in 4 N HCl:0.045% (w/v) malachite green oxalate (1:1). P_i and acid-labile phosphates appeared green on a yellow background.

AI.3.6 - Testing for contaminating microorganisms

Nonsterile granulocytes were incubated at 37°C for 24 h in 137 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 5 mM Glc, 0.5% (w/v) BSA, and 10 mM Hepes/NaOH (pH 7.4). Serial dilutions of the cell suspension were plated on LB agar and incubated overnight at 35°C. In some preparations, contaminating bacteria were detected. Colonies were homogenous and consisted of a Gram negative, motile rod, identified as *P. fluorescens* by an API NFT kit (see Results section). Single colonies were picked from the agar and were grown in LB broth at 25-30°C with shaking. All subsequent analyses were performed with broth culture stocks.

AI.3.7 - $^{32}P_i$ -radiolabeling of *P. fluorescens*

Bacteria were inoculated into LB broth (3 mL) and incubated 18 h at 25-30°C with

shaking. Cell counts were determined by a Coulter Counter, and were verified by colony forming units on LB agar. 1×10^6 bacteria were diluted into 137 mM NaCl, 5 mM KCl, 0.8 mM MgSO_4 , 5 mM Glc, 0.5% (w/v) BSA, 10 mM Hepes/NaOH (pH 7.4) (10 mL) and were preincubated at room temperature for 2 h. 50 μCi high-specific activity $^{32}\text{P}_i$ was added and the incubation continued at 37°C for 45 min. The reaction was stopped by chilling on ice and adding sodium phosphate to 1 mM. If radioactive incorporation was to be quantified, known numbers of bacteria were pipetted into microcentrifuge tubes and $\sim 5 \times 10^8$ heat-killed bacteria were added as a carrier. Bacteria were pelleted by centrifugation at $13,000 \times g$ for 5 min, washed, and counted by Cerenkov radiation in a liquid scintillation counter. The background from heat-killed carrier cells was typically 1-2% of the total cpm.

AI.3.8 - Culturing and radiolabeling of human skin fibroblasts

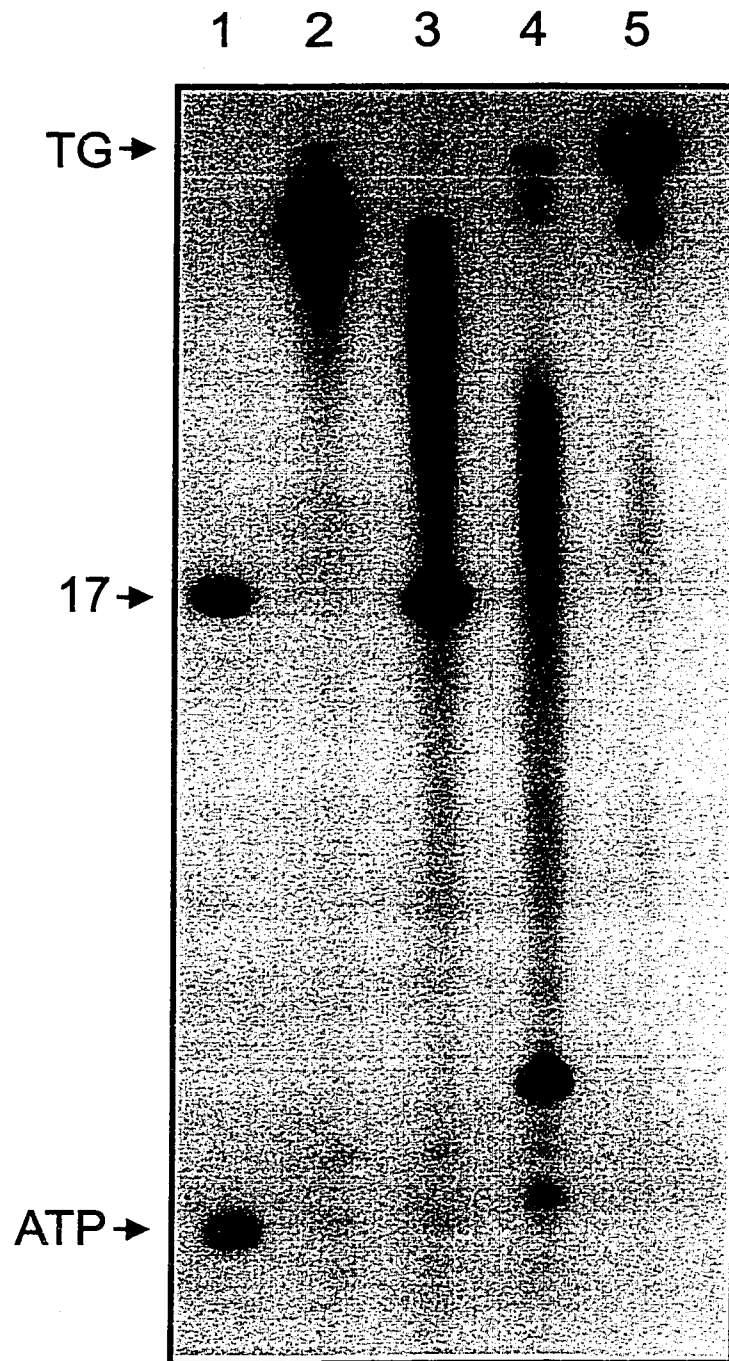
Explants of primary human fibroblasts were prepared from newborn human foreskin [25]. Cells were grown in 100×20 mm polystyrene tissue culture dishes in 10 mL DMEM containing 10% (v/v) FCS at 37°C in a humidified 5% CO_2 atmosphere. Cultures were deemed free of mycoplasmas by using both a PCR-based detection kit and Hoechst 33258 staining. Third passage cells were radiolabeled at 1-2 d post-confluency ($\sim 4 \times 10^6$ cells per plate) with 950 μCi $^{32}\text{P}_i$ per plate (final specific activity ≈ 120 mCi/mmol) for 24 h. Cells were washed, removed from the plate with trypsin/EDTA, and $[^{32}\text{P}]\text{poly(P)}$ isolated as described above. The cultures were confirmed to be free of non-fastidious organisms by incubating 50 μL of the culture medium in LB broth at 35°C for 72 h.

AI.4 - Results

When human granulocytes were labeled for 45 min with $^{32}\text{P}_i$, $82.9 \pm 1.4\%$ (mean $\pm$ SEM, $n = 8$) of the cell-associated radiolabel was pelleted at $1200 \times g$ after brief sonication in 140 mM NaCl, 1 mM CDTA, 1 mM sodium tripolyphosphate, 0.1 mM levamisole and 10 mM Hepes/NaOH (pH 7.2). Similar results were obtained using Triton X-100 detergent rather than sonication to lyse the cells. This was unexpected since granulocytes synthesize no DNA and little RNA [26,27]. The particulate radiolabel was confirmed not to be nucleic acid by its stability to nuclease digestions. Incubation with micrococcal nuclease completely degraded an exogenous ^{32}P -end-labeled oligodeoxynucleotide 17-mer, while the majority of granulocyte radiolabel ran as a higher molecular weight smear (Figure AI.2, lane 4). Slight degradation of the granulocyte radiolabel was observed with micrococcal nuclease treatment, but incubating with buffer alone produced similar results (Figure AI.2, lane 3). Apparently the radiolabel was unstable to some component of the buffer, an observation that will be addressed later in this appendix. Treatment with phosphodiesterase I retarded the mobility of the radiolabel, presumably because it bound a component of the enzyme preparation (Figure AI.2, lane 5). (In lane 5, the 17-mer was degraded to $^{32}\text{P}_i$ because of contaminating phosphomonoesterase activity.) The unknown particulate radiolabel from human granulocytes was of relatively high molecular weight when electrophoresed on 20% polyacrylamide (Figure AI.2, lane 2), but was not nucleic acid or protein. Both proteinase K used during the isolation procedure (see section AI.3.1) and nucleases failed to degrade the radiolabel.

Eventually the identity of this radiolabeled compound was established as

Figure AI.2. **Effect of nucleases on the electrophoretic mobility of Ba^{2+} -precipitable granulocyte radiolabel.** Human granulocytes were $^{32}\text{P}_i$ -labeled and $[\text{}^{32}\text{P}]\text{poly(P)}$ was isolated as detailed in section AI.3.1. Aliquots were treated as described below and were electrophoresed on a 20% polyacrylamide gel. The dried gel was exposed to x-ray film for 18 h at -100°C . The bottom half of the gel, containing $^{32}\text{P}_i$, is not shown. (*lane 1*) $[\gamma\text{}^{32}\text{P}]\text{ATP}$ + synthetic oligodeoxynucleotide (17-mer) ^{32}P -end-labeled with polynucleotide kinase; (*lane 2*) isolated $[\text{}^{32}\text{P}]\text{poly(P)}$; (*lane 3*) $[\text{}^{32}\text{P}]\text{poly(P)}$ + $[\text{}^{32}\text{P}]\text{17-mer}$ in 40 mM Tris/HCl (pH 8.8), 2.5 mM CaCl_2 incubated at 37°C for 60 min; (*lane 4*) same as lane 3 including 10 units/mL micrococcal nuclease; (*lane 5*) $[\text{}^{32}\text{P}]\text{poly(P)}$ + $[\text{}^{32}\text{P}]\text{17-mer}$ in 40 mM Tris/HCl (pH 9.5), 2.5 mM MgCl_2 and 10 units/mL phosphodiesterase I incubated at 37°C for 60 min. TG, top of gel.



[³²P]poly(P). This was determined by the following criteria.

(i) precipitation by barium acetate and acid lability

Poly(P) is known to be precipitated by Ba²⁺ [3,8,28] and is composed of phosphoanhydride linkages, which impart a characteristic lability to acid-catalyzed hydrolysis [3]. When deproteinized granulocyte extracts were treated with barium acetate, a fraction of the radioactivity was precipitated, ranging from 10-76% in different experiments. This precipitate was enriched in acid-labile ³²P (Figure AI.3, barium-ppt curve) when compared to the original extract (Figure AI.3, pre-barium curve). The time-course presented in Figure AI.3 revealed that the majority of Ba²⁺-precipitated radiolabel was hydrolyzed to ³²P_i after 5 min in 1 N HCl at 100°C. From Figure AI.3, it is apparent that a small amount of acid-stable radiolabel was also precipitated by the Ba²⁺. The identity of this acid-stable species is not known.

(ii) partial hydrolysis to a phosphate ladder

Poly(P) is known to be slowly cleaved by heating in alkali to form a continuous series differing by one or more phosphates [8]. The Ba²⁺-precipitable radiolabel (Figure AI.4, lane 2) yielded such a 'phosphate ladder' during a time-course of alkali treatment (lanes 3-8). A ladder possessing such a high electrophoretic mobility appears characteristic of low molecular weight poly(P) species. Complete alkaline hydrolysis of RNA would produce 2'(3')-nucleoside monophosphates [29] which migrate near ATP. Approximately 9 species migrating faster than ATP are present in Figure AI.4, and these co-migrate with a synthetic phosphate series (lane 9).

Figure AI.3. Time-course of acid hydrolysis of radiolabeled extracts before and after Ba^{2+} precipitation. Human granulocytes were metabolically labeled with $^{32}\text{P}_i$ and $[^{32}\text{P}]\text{poly(P)}$ was isolated from the extracts as described in section AI.3.1. Acid hydrolysis was performed on extracts prior to Ba^{2+} -precipitation (pre-barium curve) and subsequent to solubilizing Ba^{2+} - precipitates (barium-ppt curve). For each time-point, an aliquot was diluted into 300 μL ice-cold water (zero time-point only) or 300 μL ice-cold 1 N HCl. The samples were placed in boiling water for the indicated time, then immediately chilled. Acid molybdate analysis was performed as described in section AI.3.2. Both curves are means of 3 independent experiments.

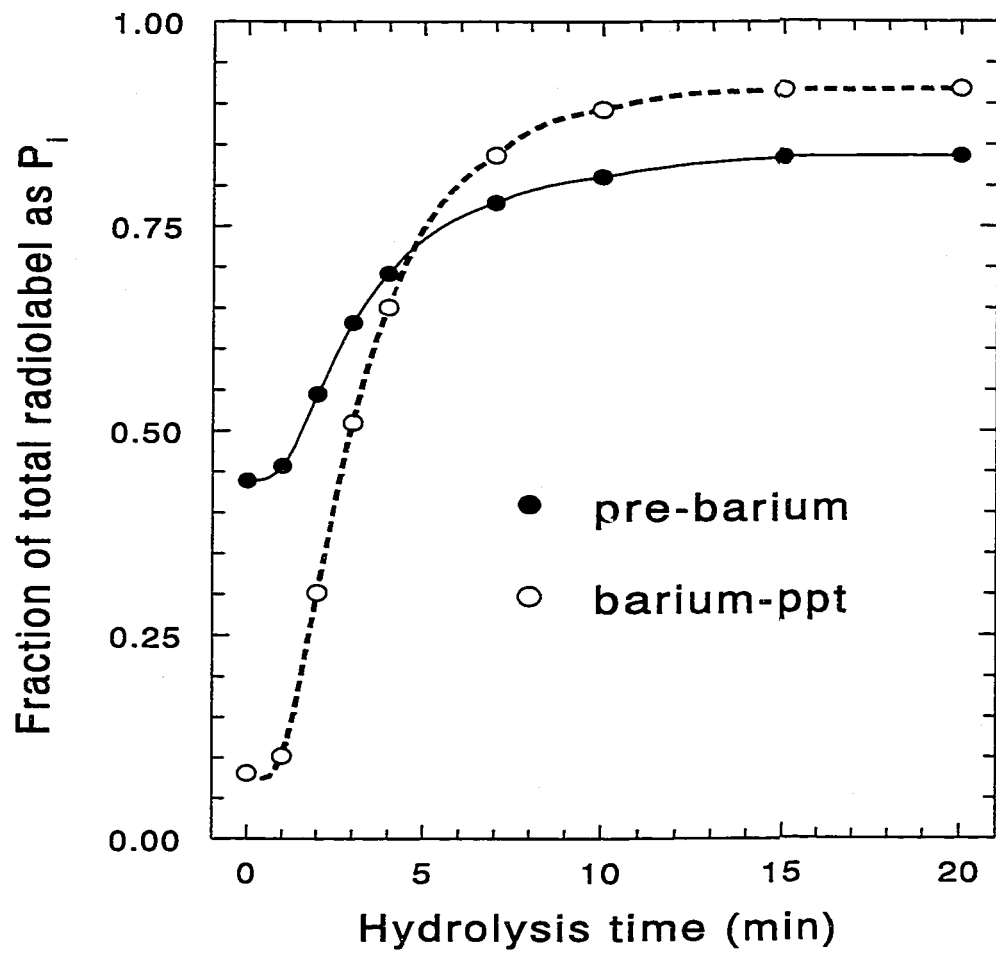
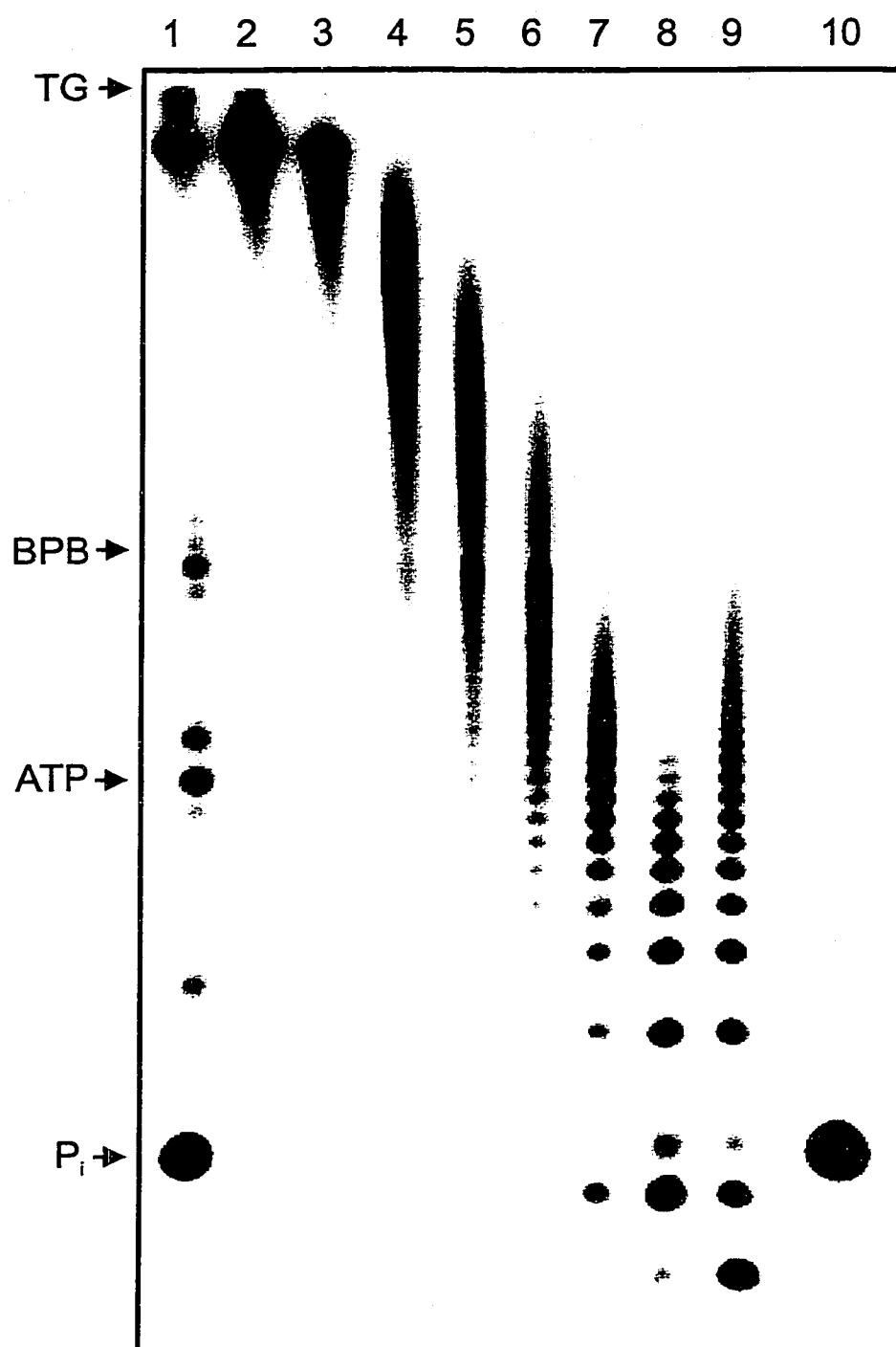


Figure AI.4. Alkaline hydrolysis of Ba²⁺-precipitable radiolabel in granulocyte extracts. Human granulocytes were ³²P_i-labeled, and [³²P]poly(P) was isolated from pooled extracts representing 7 × 10⁷ cells. Aliquots of the solubilized Ba²⁺-precipitate were treated as indicated below, cooled in ice-water, and neutralized with either HCl or NaOH. Extracts were then diluted in electrophoresis buffer and were electrophoresed on a 20% polyacrylamide gel. (*lane 1*) deproteinized granulocyte extract prior to Ba²⁺ precipitation; (*lane 2*) solubilized Ba²⁺-precipitate; (*lane 3*) solubilized Ba²⁺-precipitate heated at 100°C in 1 N KOH/1 mM CDTA for 0 min; (*lane 4*) 1 min; (*lane 5*) 2 min; (*lane 6*) 4 min; (*lane 7*) 8 min; (*lane 8*) 16 min; (*lane 9*) synthetic [³²P]poly(P); (*lane 10*) solubilized Ba²⁺-precipitate heated at 100°C in 1 N HCl for 20 min. The dried gel was exposed to x-ray film for 72 h at -100°C. BPB, bromophenol blue; TG, top of gel.



(iii) conversion to trimetaphosphate

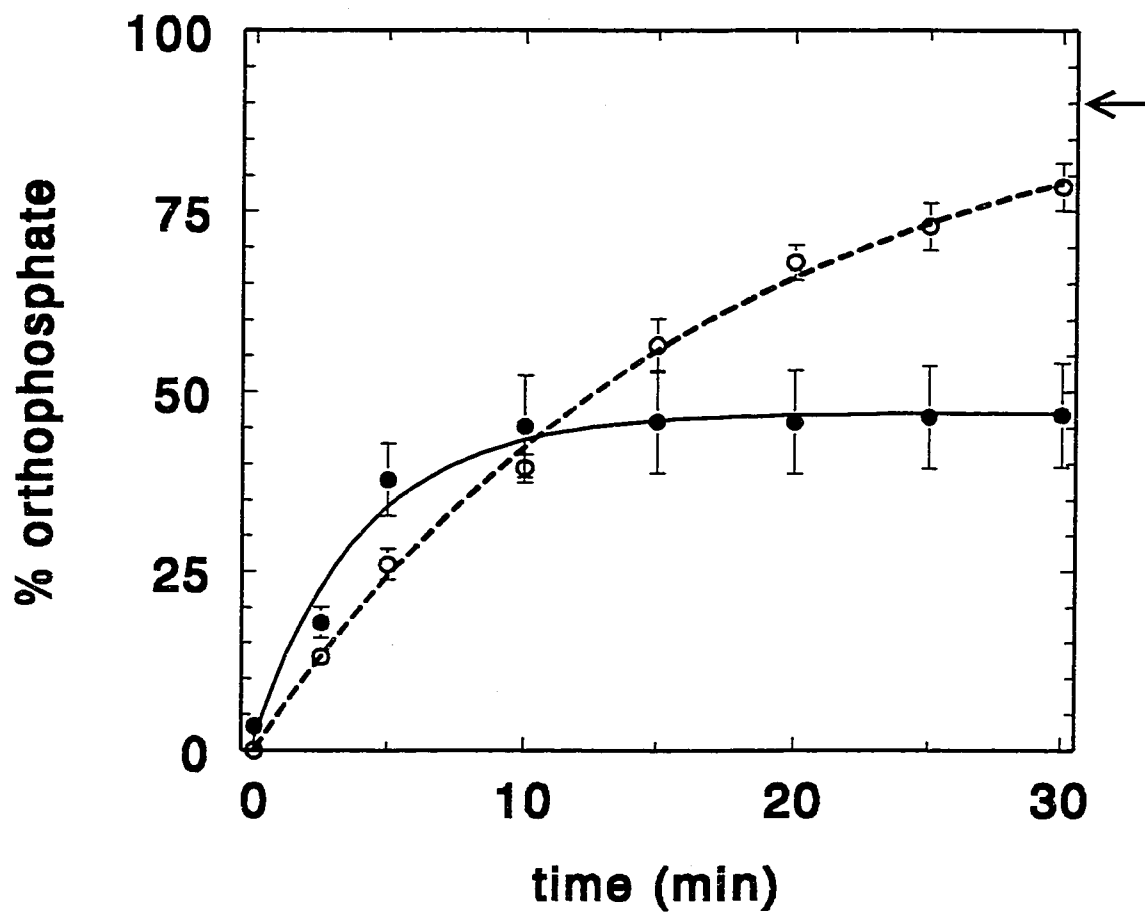
One product characteristic of the decomposition of linear polyphosphate is the cyclic trimetaphosphate [3,8]. After a 70 h incubation at 70°C, $34.5 \pm 0.8\%$ (mean $\pm$ SEM, $n = 3$) of the radiolabel from granulocytes was converted to [^{32}P]trimetaphosphate. 47% of poly(P)₅₀₀ was shown previously to be converted to trimetaphosphate with similar treatment [11].

(iv) hydrolysis by yeast exopolyphosphatase (scPPX1)

Wurst and Kornberg previously isolated an exopolyphosphatase from *S. cerevisiae* [11], which they provided as a gift. In the same publication it was reported that scPPX1 acts specifically on poly(P), processively releasing P_i until pyrophosphate remains. Significant hydrolysis was obtained when radiolabel isolated from granulocytes was treated with scPPX1 (Figure AI.5), further confirming the presence of [^{32}P]poly(P). The plateau occurring at ~50% could be explained by the presence of another unidentified radiolabeled component, or the processivity of the enzyme if ≤ 2 radioactive phosphorous were incorporated at the ends of a pre-existing poly(P) chain (i.e., the polymer became end-labeled *in vivo*). Therefore, a significant proportion of the $^{32}\text{P}_i$ incorporated into the human granulocytes was incorporated into a molecule with properties consistent with that of poly(P).

Although there have been a few sporadic reports of poly(P) in mammalian cells and tissues, the majority have been in microorganisms. I next investigated the possibility that the granulocytes were contaminated with an undetected microorganism. A single microbial species was isolated on LB agar from one granulocyte preparation. The organism was a

Figure AI.5. **Hydrolysis of poly(P) to P_i by *S. cerevisiae* exopolyphosphatase (scPPX1).** (*solid curve*) Hydrolysis of [^{32}P]poly(P) isolated from $^{32}P_i$ -labeled human granulocytes. Solubilized Ba^{2+} - precipitates were dried under vacuum then dissolved in 100 μ L exopolyphosphatase buffer (60 mM ammonium acetate, 5 mM $MgCl_2$ and 20 mM Tris/HCl (pH 7.5) [11]). Enzymatic hydrolysis was carried out at 37°C with 90 units/ μ L scPPX1. At the indicated times, samples were withdrawn into ice-cold 1 N HCl and $^{32}P_i$ determined by acid molybdate (see section AI.3.2). Total [^{32}P]poly(P) was estimated as $^{32}P_i$ after heating in 1 N HCl/100°C/10 min and is shown averaged as an arrow at 90%. (*dashed curve*) Hydrolysis of sodium phosphate glass. Reactions were performed at 37°C in exopolyphosphatase buffer containing 2.5 μ g/ μ L Type 35 sodium phosphate glass (lot #68F5060, average chain length of 31) and enzyme concentration as above. The reaction was stopped by sampling into ice-cold 1 N HCl, and P_i determined via a malachite green assay [30]. 100% P_i was estimated after heating in 1 N HCl/100°C/10 min. Both curves are means of independent experiments (*solid curve*, n = 5; *dashed curve*, n = 4) with error bars of SEM. In the absence of enzyme, no detectable hydrolysis occurred with either radiolabeled or non-radiolabeled poly(P).



Gram negative, motile rod that did not grow well at 37°C. The organism was positively identified as *P. fluorescens* (Table AI.I). Routine procedures to assess the purity of freshly isolated human granulocytes included microscopic examination. The reason for not detecting bacterial contamination earlier was investigated further. A pure culture of *P. fluorescens* was radiolabeled with $^{32}\text{P}_i$, and the radioactivity in a known number of cells was quantified by Cerenkov counting. A linear plot of radioactivity versus bacterial number yielded a slope of 1.7 Cerenkov cpm/bacterium (linear regression correlation coefficient = 0.996). This incorporation of ~2 Cerenkov cpm per bacterium is much greater than that calculated for the human granulocytes (i.e., $\sim 2 \times 10^{-3}$ Cerenkov cpm per granulocyte). Approximately 1×10^5 bacteria produced a signal equivalent to 1×10^7 granulocytes. Therefore, 1 bacterial cell was estimated to be present for every 100 human cells, a number too low to be detected by routine microscopy.

Subsequent analysis of solutions used to isolate and radiolabel granulocytes revealed the source of contamination. Bacterial counts ranging from 4.6×10^2 per mL in 137 mM NaCl, 5 mM KCl, 0.8 mM MgSO_4 , 10 mM Hepes/NaOH (pH 7.4) to 5.8×10^4 per mL in the NH_4Cl solution used during granulocyte purification [31] were observed. Although low-speed centrifugation steps were used to purify and concentrate the granulocytes, some bacteria were apparently pelleted also. *P. fluorescens* and related nonfermentative Gram negative bacteria are known to be widespread environmental and laboratory inhabitants [32], so, in retrospect, their presence was not unusual.

To my knowledge, poly(P) has not been identified in this microorganism previously. Therefore, ensuring that it had the capacity to synthesize the polymer was necessary. A pure

Table AI.I
Identification of the Contaminating Microorganism as *P. fluorescens*

The organism was isolated from a nonsterile preparation of human granulocytes as outlined in section AI.3.6. Single colonies were picked from LB agar and were subjected to tests indicated below, using the API NFT test strip for Gram-negative, nonfermentative bacteria. Biochemical tests assay for enzymatic activities, while assimilation tests determine the ability of the organism to grow on a single carbon source. Tests were performed and interpreted according to the manufacturer's instructions. The resulting numerical profile was 0156557 with a percent identification of 96.5%.

Biochemical tests		Assimilation tests	
nitrate reduction	-	D-glucose	+
tryptophanase	-	L-arabinose	-
glucose fermentation	-	D-mannose	+
arginine dihydrolase	+	D-mannitol	+
urease	-	N-acetyl-D-glucosamine	+
esculin hydrolysis	-	maltose	-
gelatinase	+	D-gluconate	+
β -galactosidase	-	caprate	+
oxidase	+	adipate	-
		L-malate	+
		citrate	+
		phenylacetate	+

culture of *P. fluorescens* was radiolabeled with $^{32}\text{P}_i$ as described in section AI.3.7. Although the organism has an optimum growth temperature of 25-30°C [32], 37°C was used to be consistent with previous data. [^{32}P]poly(P) was then isolated, and its identity verified by: (i) insolubility in barium acetate, (ii) lability to acid-catalyzed hydrolysis, (iii) hydrolysis to $^{32}\text{P}_i$ by scPPX1 [11], and (iv) formation of [^{32}P]trimetaphosphate (Figure AI.6). In the example shown in Figure AI.6, [^{32}P]poly(P) represented ~20% of the total radiolabel incorporated into the bacteria. Stationary phase *P. fluorescens* contain ~0.5 µg poly(P) per 10^9 bacteria, as determined by radiolabeling to equilibrium (LB broth, 18 h) with $^{32}\text{P}_i$ of known specific activity. The poly(P) had a length of at least 100 phosphate residues based on its mobility on polyacrylamide gels (data not shown).

Granulocytes were then isolated under strictly aseptic conditions. Except for maintaining sterility, the techniques were identical to those used previously. When 1×10^7 sterile cells were $^{32}\text{P}_i$ -labeled, the mean incorporated radiolabel was ~10-fold lower than the same number of nonsterile cells (Figure AI.7, 1-tailed Mann-Whitney nonparametric test, $p=0.05$), with a 30-fold reduction in standard deviation. [^{32}P]poly(P) could not be detected in these sterile cell extracts. Incubation of these sterile granulocytes for 24 h prior to radiolabeling had no significant effect on the incorporated radioactivity (Figure AI.7). However, a similar preincubation of nonsterile granulocytes increased incorporation by 30-fold (Figure AI.7, nonsterile bars). As much as 82% of the radiolabel in these 'aged' cells was verified to be [^{32}P]poly(P). Significant numbers of motile bacteria could be seen by microscopic examination of these nonsterile 'aged' cells. These observations strongly indicated that the [^{32}P]poly(P) signal and most of the radiolabel originated from the

Figure AI.6. Identification of [^{32}P]poly(P) in radiolabeled cultures of *P. fluorescens*. [^{32}P]poly(P) was isolated from 1×10^7 $^{32}\text{P}_i$ -labeled bacteria and was electrophoresed on a 20% polyacrylamide gel. The dried gel was exposed to x-ray film at -100°C for 48 h. *Lane 1*, solubilized Ba^{2+} precipitate; *lane 2*, same as lane 1 but heated in 1 N HCl at 100°C for 10 min; *lane 3*, same as lane 1 but incubated at 37°C for 30 min in exopolyphosphatase buffer (60 mM ammonium acetate, 5 mM MgCl_2 and 20 mM Tris/HCl (pH 7.5)) containing 200 units/ μL yeast exopolyphosphatase [11]; *lane 4*, same as lane 1 but heated at 70°C for 70 h in TMP_i buffer (0.6 mM MgCl_2 , 100 mM Tris/HCl (pH 8.0)) to generate [^{32}P]trimetaphosphate as described previously [11,33]. TG, top of gel; BPB, bromophenol blue; 3, tripolyphosphate; 2, pyrophosphate; C3, trimetaphosphate.

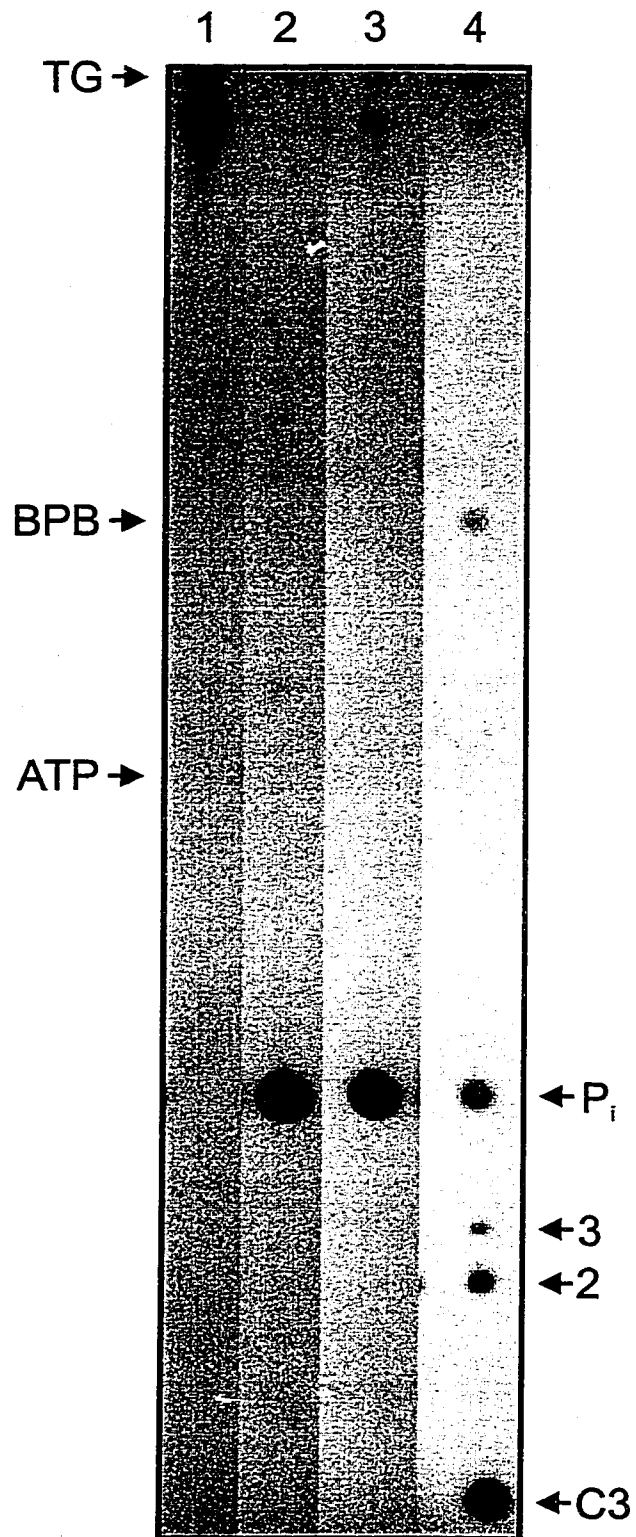
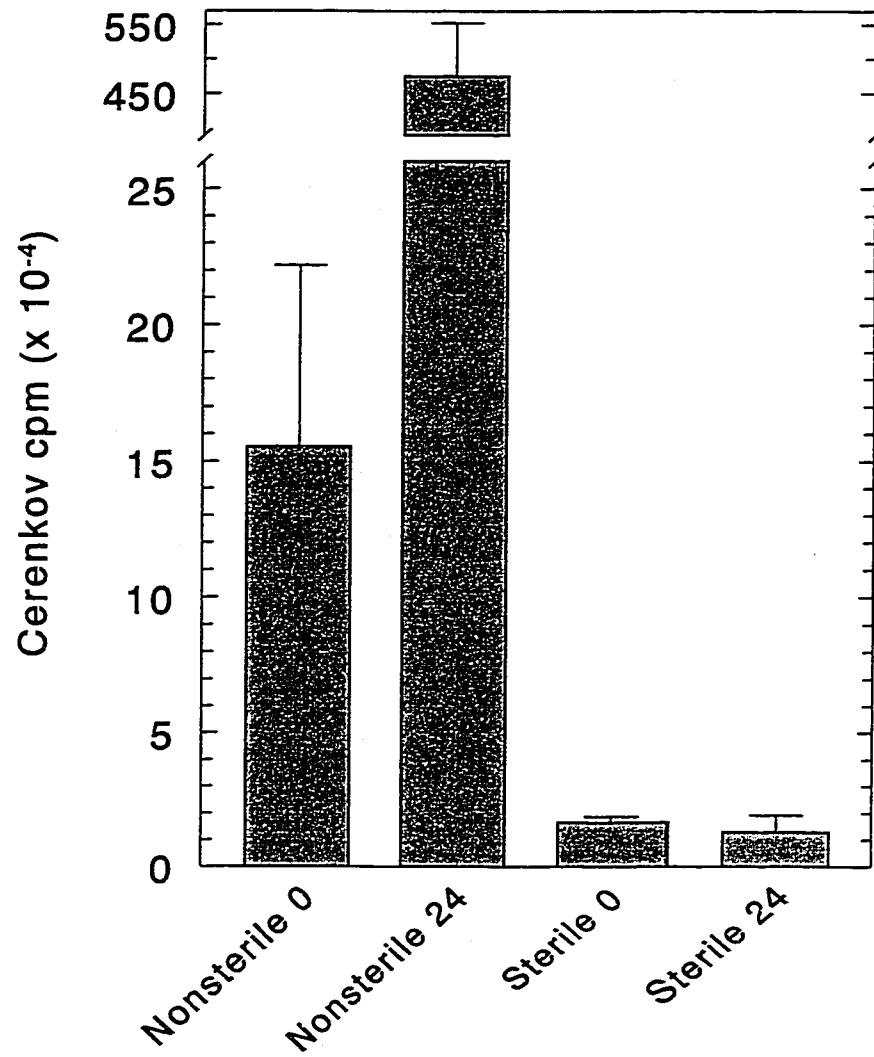


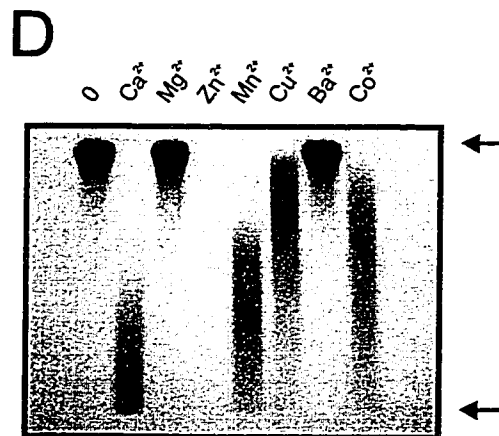
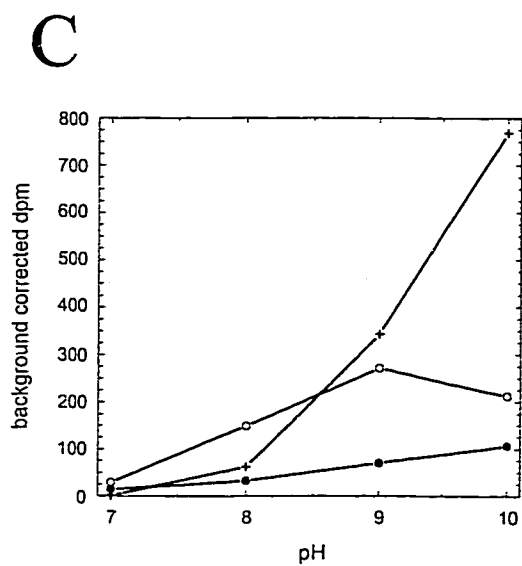
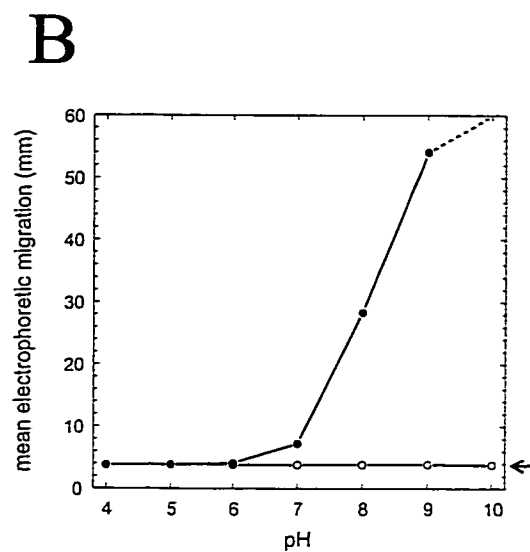
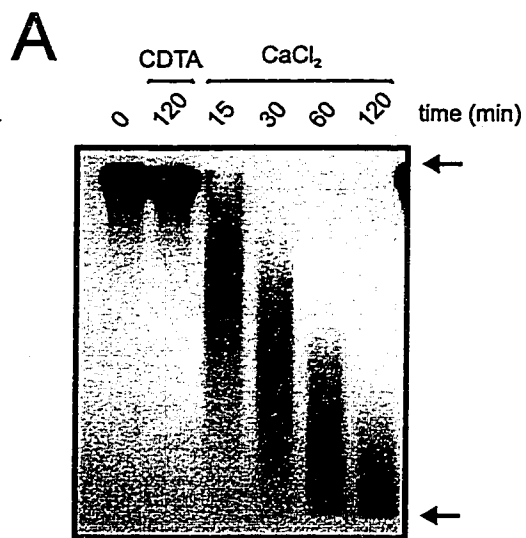
Figure AI.7. **Radiolabeling human granulocytes with [³²P]orthophosphate.** Human granulocytes (1×10^7) were radiolabeled with 50 μCi $^{32}\text{P}_i$ at 37°C for 45 min in 10 mL 137 mM NaCl, 5 mM KCl, 0.8 mM MgSO_4 , 5 mM Glc, 0.5% (w/v) BSA, and 10 mM Hepes/NaOH (pH 7.4). Cells were isolated under either sterile or nonsterile conditions, and were radiolabeled either immediately (0 h) or after 24 h preincubation at 37°C. Bars represent the means of 3 independent experiments $\pm$ SEM. The ordinate has been broken for clarity.



contaminating microorganisms and not from the granulocytes.

In lane 3 of Figure AI.2, treatment of [^{32}P]poly(P) with micrococcal nuclease buffer (containing 2.5 mM CaCl_2) partially degraded the polymer. Degradation of poly(P) is known to be accelerated by added cations [34]. Therefore, I wanted to verify that *P. fluorescens* [^{32}P]poly(P) was susceptible to degradation in the presence of Ca^{2+} . A time-dependent degradation of the polymer was observed with 2 mM CaCl_2 at pH 8 (Figure AI.8A). Poly(P) degradation is accelerated by added cation under strongly basic conditions, but is retarded under strongly acidic conditions [3,34]. To investigate the effect of pH, the electrophoretic mobility of [^{32}P]poly(P) was quantified after treatment with CaCl_2 at unit intervals between pH 4 and 10. As expected, degradation increased sharply above pH 7, but was insignificant below pH 7 (Figure AI.8B). Nonenzymic degradation of poly(P) can occur by 3 mechanisms: (i) endo-hydrolysis where an internal phosphoanhydride bond is cleaved to form two smaller poly(P), (ii) exo-hydrolysis where P_i is liberated sequentially from the ends of the polymer, and (iii) metaphosphate formation where small cyclic phosphates (especially trimetaphosphate) are formed internally [3,34]. All 3 types of degradation increased above pH 7 (Figure AI.8C). In panel C, pentapolyphosphate was used as a reliable measure of endo-hydrolysis because it cannot be formed from the degradation of common cyclic structures such as trimeta- and tetrametaphosphate [34]. Other small molecular weight linear poly(P) from $n=2$ to $n=12$ were also visible in the TLC system (data not shown). Taking this into consideration, endo-hydrolysis represented the vast majority of Ca^{2+} -catalyzed degradation above pH 7. Finally, a qualitative analysis was performed to assess the relative ability of various divalent cations to catalyze degradation of [^{32}P]poly(P). After 60 min at

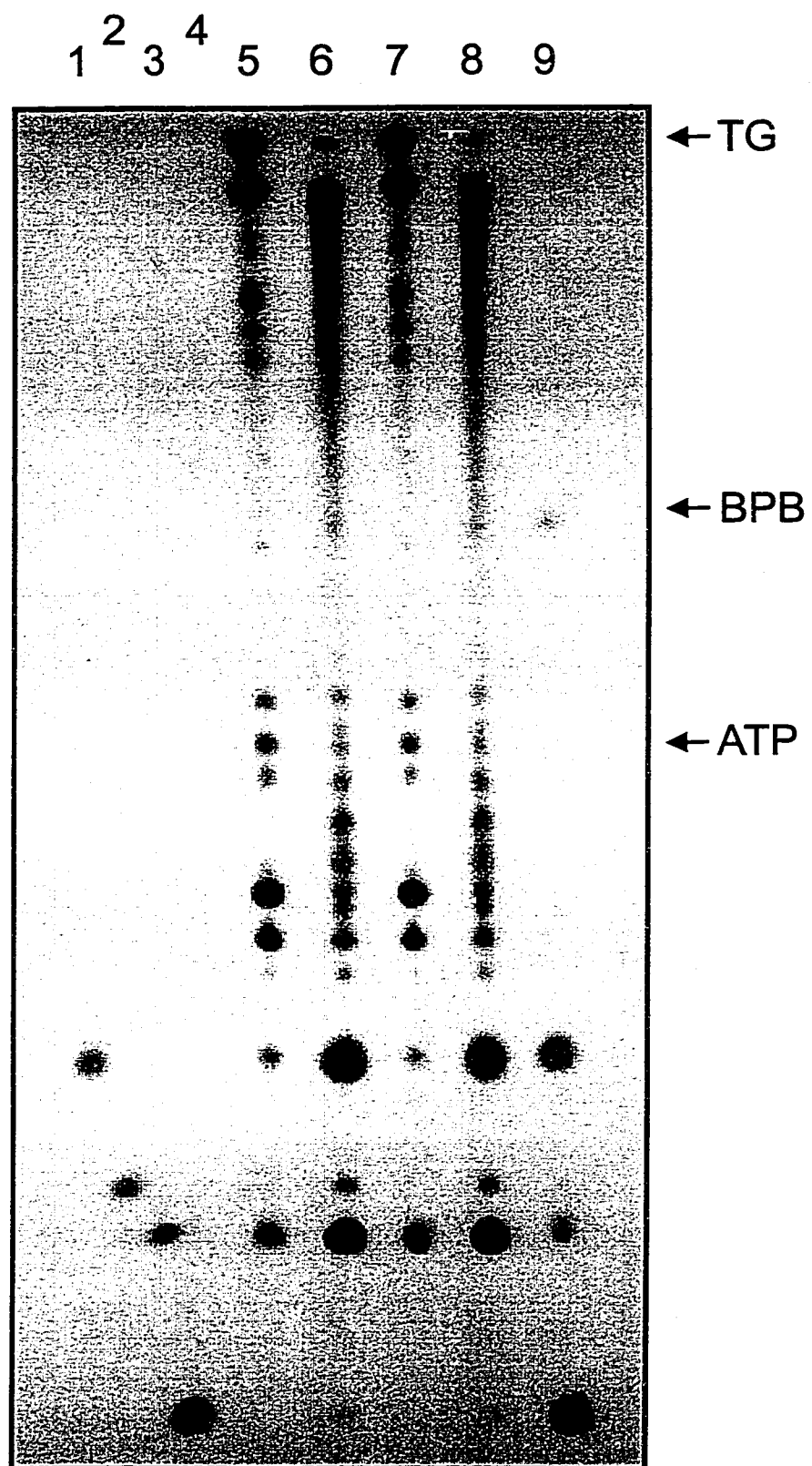
Figure AI.8. **Divalent cation-catalyzed degradation of [^{32}P]poly(P).** [^{32}P]poly(P) was isolated from $^{32}\text{P}_i$ -labeled *P. fluorescens*. Panel A. Ca^{2+} catalyzes a time-dependent degradation of [^{32}P]poly(P). [^{32}P]poly(P) was incubated in 40 mM Tris/HCl (pH 8.0) with either 2 mM CaCl_2 or 10 mM CDTA at 37°C for the indicated amount of time. Samples were then electrophoresed on a 12% polyacrylamide gel. This figure is representative of 2 independent experiments. *Arrowheads* mark the top and bottom of the gel respectively. Panel B. Ca^{2+} -catalyzed degradation of [^{32}P]poly(P) is pH dependent. [^{32}P]poly(P) was incubated at 37°C in 20 mM acetic acid, 20 mM pyridine, 20 mM triethanolamine and 20 mM ethanolamine (standardized to indicated pH with HCl or NaOH), containing either 2 mM CaCl_2 (-●-) or 2 mM CDTA (-○-) for 20 min. Samples were electrophoresed on a 12% polyacrylamide gel. Each lane of the dried gel was cut into 10 equal pieces (6 mm each), which were counted individually with scintillation fluid in a liquid scintillation counter. A distribution plot of cpm vs. distance migrated was obtained for each pH and the mean of the distribution estimated by manual calculation. The distribution mean vs. pH is plotted. A value could not be calculated for Ca^{2+} , pH 10 since all of the radioactivity migrated off the gel. The curve is *dashed* to highlight this fact. An *arrow* depicts the mean migration of the original [^{32}P]poly(P) sample. Panel C. Increased Ca^{2+} -catalyzed degradation of [^{32}P]poly(P) between pH 7 and pH 10 is the result of increased endo-hydrolysis. [^{32}P]poly(P) was incubated at 37°C for 60 min in 20 mM acetic acid, 20 mM pyridine, 20 mM triethanolamine, 20 mM ethanolamine and 2 mM CaCl_2 (standardized to indicated pH). Samples were spotted on a prewashed PEI-cellulose TLC sheet, chromatographed with 0.6 M NH_4HCO_3 , 4 mM CDTA, and visualized by overnight autoradiography. Orthophosphate (-●-, $R_f \approx 0.75$), trimetaphosphate (-○-, $R_f \approx 0.65$) and pentapolyphosphate (-✚-, $R_f \approx 0.30$) were cut from the sheet and were counted individually in a liquid scintillation counter with scintillation fluid. Depicted curves are means of 2 experiments. Panel D. Degradation of [^{32}P]poly(P) catalyzed by various divalent cations. [^{32}P]poly(P) (*lane 0*) was incubated in 40 mM Tris/HCl (pH 8.0), containing 2 mM CaCl_2 , MgCl_2 , ZnSO_4 , MnCl_2 , CuSO_4 , barium acetate, or CoCl_2 at 37°C for 60 min. Samples were then electrophoresed on a 12% polyacrylamide gel. This figure is representative of 2 independent experiments. *Arrowheads* mark the top and bottom of the gel respectively.



pH 8, all tested cations had degraded the polymer, except for Mg^{2+} and Ba^{2+} (Figure AI.8D). Degradation in the presence of Zn^{2+} was so severe that all the radiolabel migrated off the gel. The observed rates of catalysis are ranked as follows: $\text{Zn}^{2+} > \text{Ca}^{2+} > \text{Mn}^{2+} > \text{Co}^{2+} \geq \text{Cu}^{2+}$.

Others have demonstrated previously that subcellular fractions of mammalian cells (e.g., human fibroblast lysosomes [8] and rat liver nuclei [6,7]) incorporated $^{32}\text{P}_i$ into poly(P). Considering the problems of microbial contamination described above, the presence of poly(P) in cultured human fibroblasts was readdressed. Radiolabeling of intact primary human skin fibroblasts was deemed the best option for detecting the polymer. Fibroblasts were metabolically labeled with $^{32}\text{P}_i$ (950 μCi per plate) in tissue culture media for 24 h. Only ~1% of the total incorporated radiolabel from the cell extracts was precipitable by Ba^{2+} (Figure AI.9, lanes 5 and 7). The formation of trimetaphosphate is considered a reliable test for the presence of poly(P) [3,8]. A small amount of [^{32}P]trimetaphosphate was formed from the Ba^{2+} -precipitates (Figure AI.9, lanes 6 and 8), representing ~1 pmole of phosphate per 10^6 cells. Although it has been reported previously that mammalian tissues contain tiny quantities of poly(P) [20], the small amounts of [^{32}P]trimetaphosphate observed in Figure AI.9 may not have originated from inorganic poly(P). In all cases analyzed, heating inorganic poly(P) at 70°C for 70 h in TMP_i buffer generated a ratio of trimetaphosphate:tripolyphosphate > 1 (e.g., see Figure AI.6 lane 4, and Figure AI.9 lane 9). This occurred with both high molecular weight and low molecular weight, synthetic [^{32}P]poly(P) with an average chain length of ~10 phosphates (data not shown). However, the radiolabel isolated from fibroblast extracts generated trimetaphosphate and tripolyphosphate in a ratio < 1 (Figure AI.9, lanes 6 and 8). Low yields of trimetaphosphate

Figure AI.9. Inorganic [^{32}P]poly(P) cannot be detected in extracts from $^{32}\text{P}_i$ -labeled human skin fibroblasts. Primary fibroblasts were radiolabeled for 24 h with 950 $\mu\text{Ci } ^{32}\text{P}_i$, and poly(P) was isolated as described in section AI.3.1. Radiolabel was analyzed by electrophoresis on a 20% polyacrylamide gel. *Lane 1*, [^{32}P]orthophosphate; *lane 2*, [^{32}P]tripolyphosphate; *lane 3*, [^{32}P]pyrophosphate; *lane 4*, [^{32}P]trimetaphosphate; *lane 5*, solubilized Ba^{2+} -precipitate from radiolabeled fibroblast culture; *lane 6*, same as lane 5, except heated at 70°C for 70 h in TMP_i buffer (0.6 mM MgCl_2 , 100 mM Tris/HCl (pH 8.0)) to generate trimetaphosphate as described previously [11,33]; *lanes 7 and 8*, replicate experiments for lanes 5 and 6, respectively; *lane 9*, [^{32}P]poly(P) from *P. fluorescens* heated at 70°C for 70 h in TMP_i buffer. Synthetic markers (lanes 2-4) were formed by heating synthetic [^{32}P]poly(P) as indicated above, and then purifying each species on PEI-cellulose TLC [24,33]. TG, top of gel; BPB, bromophenol blue.



are characteristic of very small poly(P) of 5 or 6 residues [34]. Since there were no such species on the 20% gel (Figure AI.9, lanes 5 and 7), the trimetaphosphate and tripolyphosphate could have originated from a low molecular weight organic poly(P) compound. Possible candidates are discussed below. Therefore, using the most stringent tests available, I could not detect [^{32}P]poly(P) in human fibroblast extracts with certainty.

AI.5 - Discussion

The apparent presence of [^{32}P]poly(P) in radiolabeled human granulocytes led to the detection of a minor contamination of *P. fluorescens* that was undetectable by routine microscopy. The bacteria existed at low numbers in nutrient-limited solutions used to isolate and radiolabel the granulocytes. Although these solutions were filter-sterilized at time of preparation, they were not stored and handled aseptically thereafter. It was believed, incorrectly, that stringently sterile techniques were not necessary since $^{32}\text{P}_i$ -radiolabeling of granulocytes was short-term. Granulocytes possess anti-bacterial activity from both O_2 -dependent and -independent mechanisms [35,36]. Bacterial components such as fMLP and LPS will influence signal transduction mechanisms, and will alter the physiology of these cells [37,38]. Therefore, maintaining stringent sterility is important, and such conditions were undertaken in all subsequent experiments presented in this thesis. Granulocytes were isolated thereafter in a laminar flowhood with sterile solutions that had been stored and handled aseptically.

As is evident in Figure AI.7, sterile human granulocytes did assimilate a small amount of radiolabel. Assuming ~50% Cerenkov counting efficiency, 1×10^7 granulocytes

incorporated only ~0.03% of the 50 μCi of high-specific activity $^{32}\text{P}_i$ available. These cells are known to have very low rates of nucleic acid synthesis [26,27], which could explain the modest radiolabeling. Thus, a *P. fluorescens* contamination representing 1/1000 the granulocyte count would be significant. Since other cultured cells incorporate $^{32}\text{P}_i$ at greater rates, microbial contamination might not be as evident. However, some identifications of ^{32}P]poly(P) in mammalian cells have used $^{32}\text{P}_i$ -labeling of subcellular fractions, such as isolated nuclei [7,8,28,39]. These organelles are not expected to retain the capacity to synthesize high-energy bonds, increasing the significance of any ^{32}P]poly(P) from a minor contaminating microbe. Certain biochemical solutions (e.g., those used to isolate subcellular organelles) pose additional risks. Because they are nutrient-limited, low numbers of microorganisms can exist undetected and microbial poly(P) may be preferentially synthesized under these unfavourable growth conditions [3].

No poly(P) could be detected reliably in fibroblasts after labeling with $^{32}\text{P}_i$ for 24 h. The very small amounts of ^{32}P]trimetaphosphate and ^{32}P]tripolyphosphate detected on 20% PAGE could have originated from some unknown organic poly(P) compound. Formation of tripolyphosphate requires the presence of at least 3 consecutive phosphoanhydride bonds, while trimetaphosphate formation requires at least 4 [34]. Nucleoside triphosphates and the 5'-cap of mRNA are not candidates since they have only 2 such bonds. Mammalian cells can contain small quantities of adenosine 5'-tetraphosphate [40], diadenosine 5',5'-P₁,P₅-pentaphosphate, and diadenosine 5',5'-P₁,P₆-hexaphosphate [41]. Yeast cells have been shown to contain adenosine 5'-pentaphosphate [42], although to my knowledge this molecule has yet to be found in a mammalian cell. All can form tripolyphosphate when degraded, and

all but adenosine tetraphosphate can potentially form trimetaphosphate. Whether these species were present on 20% PAGE is not known with certainty. However, based on the mobility of a minor contaminant in commercial preparations of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, adenosine tetraphosphate is probably one of the prominent bands situated between ATP and P_i in Figure AI.9, lanes 5 and 7. The second prominent band could be one of the other aforementioned nucleotide derivatives.

It is not possible to completely rule out that tiny amounts of poly(P) exist in mammalian cells, possessing a function different from that in microorganisms. Using the highly specific enzymes *E. coli* poly(P) kinase and *S. cerevisiae* exopolyphosphatase, Kumble and Kornberg recently identified low levels of poly(P) in rodent tissues (25-120 μM P_i residues in chains of 50-800) [5]. Using the same techniques, the authors also detected $[\text{}^{32}\text{P}]\text{poly(P)}$ in $^{32}\text{P}_i$ -radiolabeled cell lines of fibroblasts, T-cells, kidney and adrenal cells. Although poly(P) were present in various subcellular fractions (plasma membranes, microsomes, mitochondria, and nuclei), the authors were not successful detecting $[\text{}^{32}\text{P}]\text{poly(P)}$ by radiolabeling subcellular fractions (Kumble and Kornberg, personal communication). My results and those of Kumble and Kornberg call into question previous identifications of $[\text{}^{32}\text{P}]\text{poly(P)}$ in $^{32}\text{P}_i$ -labeled rat liver nuclei and human fibroblast lysosomes [6-8]. Considering the problems presented in this appendix, I strongly recommend that stringent aseptic procedures be used in any future studies of poly(P) in higher organisms. If $[\text{}^{32}\text{P}]\text{poly(P)}$ is detected after $^{32}\text{P}_i$ -labeling intact mammalian cells or subcellular fractions, there is a simple test to confirm its origin. Mammalian cells and organelles are lysed by treating with mild, non-denaturing detergents (e.g., $\leq 0.5\%$ Triton X-100), while many

contaminating microbes remain viable. If [^{32}P]poly(P) is still synthesized post-lysis, then the polymer likely originated from a contaminating microorganism.

AI.6 - References

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Appendix II

**Percentage of ^3H -RNA expected in nuclear and cytoplasmic fractions
after a 6 h pulse with [^3H]uridine. Theoretical Calculations.**

AII.1 - Using values obtained by Sociero *et al.* [1] (distribution of radiolabeled RNA and orcinol-estimated RNA in subcellular fractions of HeLa cells)

$$\text{percentage of total cell RNA as mRNA} = 3.0\%$$

$$\text{percentage of total cell RNA as hnRNA} = 1.7\%$$

Assuming a value of 26 pg total RNA per cell [2]:

$$\text{mass of mRNA per cell} = 0.78 \text{ pg}$$

$$\text{mass of hnRNA per cell} = 0.44 \text{ pg}$$

The half-life of hnRNA has been estimated to be ~23 min [2]. Therefore, in a 6 h pulse, hnRNA will be radiolabeled to equilibrium. The half-life of mRNA has been estimated to be 10 h [3]. To maintain mRNA levels in a 6 h pulse, the mass of radiolabeled mRNA (per cell) is predicted to be:

$$(\text{mass mRNA}) - (\text{mass mRNA} \times 2^{-\text{time/half-life}}) = 0.78 - 0.78 \times 2^{-6/10} = 0.27 \text{ pg}$$

Therefore, the percentage of radiolabeled pol II transcripts expected to be found in the cytoplasm after a 6 h pulse of [³H]uridine is:

$$0.27 / (0.27 + 0.44) \times 100\% = 38\%$$

AII.2 - Using values obtained by Brandhorst and McConkey [2] (distribution of radiolabeled RNA in subcellular fractions of mouse L cells)

$$\text{percentage of total cell RNA as mRNA} = 3.0\% [1]$$

$$\text{percentage of total cell RNA as hnRNA} = 6.9\%$$

Assuming a value of 26 pg total RNA per cell:

$$\text{mass of mRNA per cell} = 0.78 \text{ pg}$$

$$\text{mass of hnRNA per cell} = 1.79 \text{ pg}$$

For reasons described in section AII.1, the mass of radiolabeled mRNA (per cell) is predicted to be 0.27 pg after a 6 h pulse.

Therefore, the percentage of radiolabeled pol II transcripts expected to be found in the cytoplasm after a 6 h pulse of [³H]uridine is:

$$0.27 / (0.27 + 1.79) \times 100\% = 13\%$$

AII.3 - Using values obtained by Schibler *et al.* [4] (R_{ot} analysis of nuclear and cytoplasmic RNA fractions in MPC-11 cells)

Let y represent the % driver matching probe sequence. Using R_{ot} values obtained when [³²P]cDNA (made from poly(A)⁺ mRNA) was annealed to poly(A)⁺ mRNA or total nuclear RNA:

$$\begin{aligned} \text{cytoplasmic (Crt}_{1/2} \times y) &= \text{nuclear (Crt}_{1/2} \times y) \\ 3.07 \times 100\% &= 412.5 \times y_{\text{nuclear}} \\ \therefore y_{\text{nuclear}} &= 0.74\% \end{aligned}$$

Assume that the average hnRNA is 4× larger than its spliced mRNA [5]. Therefore, $4 \times 0.74\% \approx 3\%$ of nuclear RNA mass is hnRNA.

$$\begin{aligned} \text{mass of total RNA per cell} &= 20 \text{ pg} \\ \text{mass of cytoplasmic mRNA per cell} &= 20 \text{ pg} \times 1.5\% \\ &= 0.3 \text{ pg} \end{aligned}$$

$$\begin{aligned}\text{mass of nuclear hnRNA per cell} &= 20 \text{ pg} \times 10\% \times 3\% \\ &= 0.06 \text{ pg}\end{aligned}$$

The half-life of hnRNA has been estimated to be ~23 min [2]. Therefore, in a 6 h pulse, hnRNA will be radiolabeled to equilibrium. The half-life of mRNA has been estimated to be 10 h [3]. To maintain mRNA levels in a 6 h pulse, the mass of radiolabeled mRNA (per cell) is predicted to be:

$$(\text{mass mRNA}) - (\text{mass mRNA} \times 2^{-\text{time/half-life}}) = 0.3 - 0.3 \times 2^{-6/10} = 0.10 \text{ pg}$$

Therefore, the percentage of radiolabeled pol II transcripts expected to be found in the cytoplasm after a 6 h pulse of [³H]uridine is:

$$0.10 / (0.10 + 0.06) \times 100\% = 63\%$$

AII.4 - Using values obtained by Muralidhar and Johnson [6] (distribution of radiolabeled RNA determined by subcellular fractionation and hybridization to specific cDNA probes)

The authors analyzed the incorporation of [³H]uridine into various nuclear and cytoplasmic RNA species over a period of 150 min. DHFR, TS, ribosomal protein L30 and L32, and two abundant but unknown species denoted M7 and R11 were studied. Radiolabel in exon sequences was analyzed since intron sequences were removed by ribonuclease digestion after hybridization to a cDNA probe. Therefore, corrections must be made to account for this intron material in hnRNA.

Table AII.I

hnRNA and mRNA sizes of the 6 mouse genes analyzed by Muralidhar and Johnson [6].

Gene	hnRNA Size (bases)	mRNA Size (bases)	Ratio of hnRNA to mRNA Size	Reference
DHFR	31 000	1600	19.4	GenBank ID SEQ_MUSFOL
TS	12 000	1100	10.9	GenBank ID SEQ_MUSTHYS
L30	2711	463	5.9	GenBank Accession #K02928
L32	3225	488	6.6	GenBank Accession #K02060
M7	unknown	unknown	4	[5]
R11	unknown	unknown	4	[5]

Plots of radiolabel incorporation into nuclear and cytoplasmic exon species up to 150 min were illustrated. Extrapolating these plots and taking into consideration the intron sequences that were removed from hnRNA during ribonuclease digestion, the amount of radiolabel in both cytoplasmic and nuclear RNA at 6 h can be estimated.

Table AII.II
Radioactivity estimated to be present in hnRNA and mRNA after a 6 h pulse of [3H]uridine.

Gene Name	CPM ($\times 10^{-3}$)			
	mRNA	nonamplified mRNA*	hnRNA	nonamplified hnRNA*
DHFR	7.7	0.03	19.2	0.06
TS	2.9	0.06	3.4	0.07
L30	0.4	0.4	0.3	0.3
L32	0.2	0.2	0.7	0.7
M7	6.5	6.5	1.7	1.7
R11	4.8	4.8	4.0	4.0
Sum	22.5	12.0	29.3	6.8

* 3T6 cell-lines used overexpressed DHFR and TS by factors of 300 and 50 respectively

Therefore,

% total cytoplasmic

$$\begin{aligned} \text{radiolabel at 6 h} &= (22.5) / (22.5 + 29.3) \times 100\% \\ &= 43\% \end{aligned}$$

and

% total nonamplified

cytoplasmic radiolabel

$$\begin{aligned} \text{at 6 h} &= (12.0) / (12.0 + 6.8) \times 100\% \\ &= 64\% \end{aligned}$$

The first percentage (i.e., 43%) likely biases toward rare messages, while the second biases toward more abundant mRNA. Which of the two values is more representative of the actual value is not known, so I will state that the calculated value is somewhere between 43 and 64%.

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Appendix III
Suppliers of Reagents

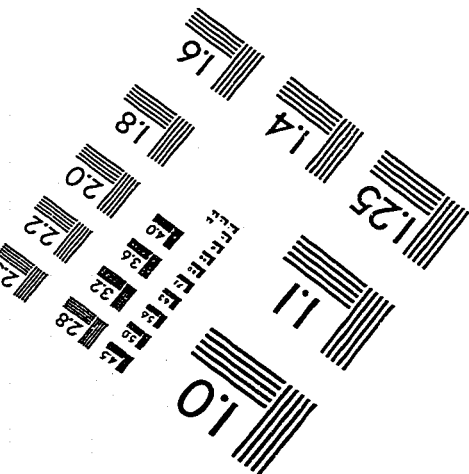
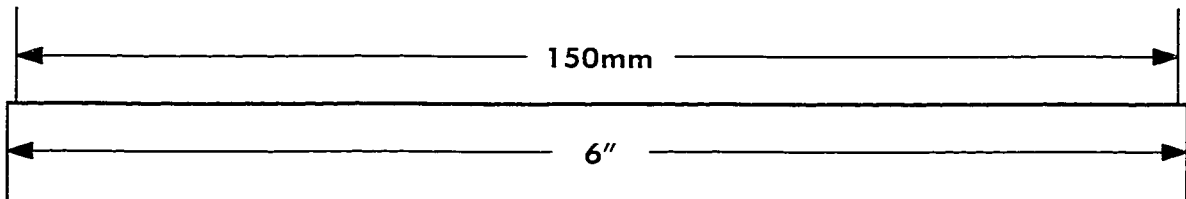
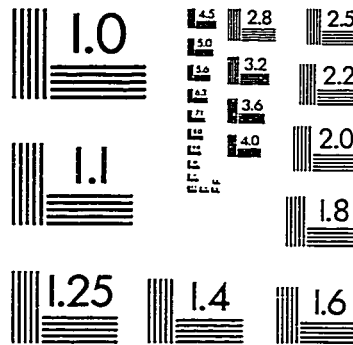
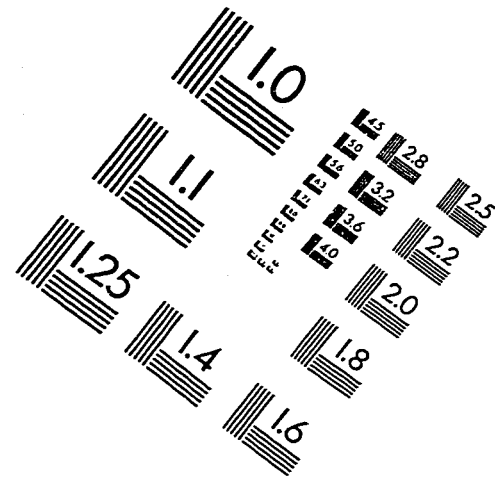
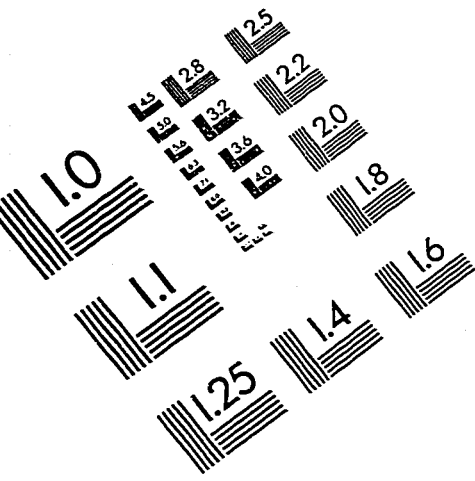
<u>REAGENT</u>	<u>SUPPLIER</u>	<u>LOCATION</u>
actinomycin D	Life Technologies Inc.	Burlington, ON
α -amanitin	Sigma-Aldrich Canada Ltd.	Oakville, ON
ampholytes, Pharmalyte 3-10	Pharmacia Biotech Inc.	Québec
ampholytes, Bio-Lyte 5/7	Bio-Rad Laboratories Canada Ltd.	Mississauga, ON
antibody, alkaline phosphatase conjugated secondary	Sigma-Aldrich Canada Ltd.	Oakville, ON
antibody, anti-Gst π	Biotrin International Ltd.	Dublin, Ireland
antibody, anti-HA	Boehringer Mannheim	Laval, PQ
antibody, anti-p27Kip1	Santa Cruz Biotechnology	California
antibody, Texas Red conjugated secondary	Cedarlane Laboratories Ltd.	Hornby, ON
API NFT kit	bioMérieux Vitek Inc.	Hazelwood, MO
bacteriophage M13K07	Stratagene Cloning Systems	La Jolla, CA
CDTA	Sigma-Aldrich Canada Ltd.	Oakville, ON
cell-lines	American Type Culture Collection	Rockville, MD
cycloheximide	Sigma-Aldrich Canada Ltd.	Oakville, ON
Dektol developer	Eastman Kodak Co.	Rochester, NY
Diff-Quik	VWR Canlab	Mont-Royal, PQ
DNA random priming kit	Amersham Life Science Inc.	Oakville, ON

En ³ Hance	DuPont NEN Research Products	Mississauga, ON
<i>Escherichia coli</i> strains	Stratagene Cloning Systems	La Jolla, CA
fetal calf serum	ImmunoCorp	Montréal, PQ
fluorescamine	Sigma-Aldrich Canada Ltd.	Oakville, ON
Freund's adjuvant	Difco Laboratories	Detroit, MI
glycogen	Boehringer Mannheim	Laval, PQ
GM-CSF	Sandoz Canada Inc.	Dorval, PQ
Hypercoat LM-1	Amersham Life Science Inc.	Oakville, ON
I.M.A.G.E. clones	Research Genetics Inc.	Alabama
levamisole	Sigma-Aldrich Canada Ltd.	Oakville, ON
LipofectAmine	Life Technologies Inc.	Burlington, ON
Lympholyte-H	Cedarlane Laboratories Ltd.	Hornby, ON
markers, DNA	Pharmacia Biotech Inc.	Québec
markers, protein	Life Technologies Inc.	Burlington, ON
markers, RNA	Boehringer Mannheim	Laval, PQ
media, DMEM	Life Technologies Inc.	Burlington, ON
media, RPMI 1640	Life Technologies Inc.	Burlington, ON
membranes, Hybond-N and N ⁺	Amersham Life Science Inc.	Oakville, ON
membranes, Immobilon-P	Millipore Corporation	Bedford, MA
micro-electroporation chamber	Life Technologies Inc.	Burlington, ON

microscope slides, polylysine coated	VWR Canlab	Mont-Royal, PQ
oligodeoxynucleotides (synthetic)	Life Technologies Inc.	Burlington, ON
oligo(dT) cellulose	Pharmacia Biotech Inc.	Québec
pancreatic microsomes	Boehringer Mannheim	Laval, PQ
PCR mycoplasma detection kit	Stratagene Cloning Systems	La Jolla, CA
PEI-cellulose TLC sheets	Mandel Scientific Co. Ltd.	Guelph, ON
Permout	Fisher Scientific Co.	Nepean, ON
PicoGreen	Molecular Probes Inc.	Eugene, Oregon
placental ribonuclease inhibitor	Life Technologies Inc.	Burlington, ON
protein A-Sepharose	Sigma-Aldrich Canada Ltd.	Oakville, ON
QIAquick gel extraction kit	Qiagen Inc.	Mississauga, ON
rabbit reticulocyte and wheat germ lysates	Promega	Madison, WI
radioactive isotopes	Amersham Life Science Inc.	Oakville, ON
retinoic acid, all <i>trans</i>	Sigma-Aldrich Canada Ltd.	Oakville, ON
ribonucleoside-vanadyl complexes	New England Biolabs	Beverly, MA
scintillation fluid	VWR Canlab	Mont-Royal, PQ
Sequenase Version 2.0 DNA sequencing kit	United States Biochemical Corporation	Cleveland, Ohio
silicone oil	VWR Canlab	Mont-Royal, PQ

Spectra/Gel cation exchange resin 50X8	Spectrum Medical Industries Inc.	Los Angeles, CA
sodium phosphate glass (type 35)	Sigma-Aldrich Canada Ltd.	Oakville, ON
sodium trimetaphosphate and tripolyphosphate	Sigma-Aldrich Canada Ltd.	Oakville, ON
Superscript RT, buffer and DTT	Life Technologies Inc.	Burlington, ON
T7 & T3 promoter primers	Promega	Madison, WI
T7 Cap-Scribe	Boehringer Mannheim	Laval, PQ
Triton X-114	Sigma-Aldrich Canada Ltd.	Oakville, ON
Ultrafree-MC Durapore microcentrifuge filter	Millipore Corporation	Bedford, MA
V8 protease	Sigma-Aldrich Canada Ltd.	Oakville, ON
Vacutainers	Becton Dickinson VACUTAINER Systems	Franklin Lakes, NJ
vector, pBluescript	Stratagene Cloning Systems	La Jolla, CA
vector, pcDNA3	Invitrogen	Carlsbad, CA
XOMAT AR x-ray film	Eastman Kodak Co.	Rochester, NY

IMAGE EVALUATION TEST TARGET (QA-3)



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