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CHARACTERIZATION OF LAMINS A AND C EXPRESSION DURING DIFFERENTIATION OF HL-60 CELLS TOWARDS MONOCYTES/MACROPHAGES *IN VITRO*

by

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To My Family

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ABBREVIATIONS

DMEM:	Dulbecco's Modified Eagle Medium
DMSO:	Dimethyl Sulfoxide
FITC:	Fluorescein Isothiocyanate
IFM:	Immunofluorescence Microscopy
IFP:	Intermediate Filament Protein
PBS:	Phosphate Buffered Saline
PMA:	Phorbol 12-Myristate 13-Acetate, or TPA
PRISTANE:	2,6,10,14-Tetramethylpentadecane
SDS-PAGE:	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TPA:	Phorbol 12-Myristate Acetate, or PMA
TR:	Transferrin Receptor

ABSTRACT

The inducible human promyelocytic cell line HL-60 is known to express a limited complement of intermediate filament proteins (IFPs). In the undifferentiated state these cells express B-type nuclear lamins, but lack lamins A and C and the cytoplasmic IFP vimentin (Paulin-Levasseur et al., 1988). In response to TPA, an inducer of differentiation towards macrophages, or to DMSO, which promotes maturation along the granulocytic path, HL-60 cells express the full complement of IFP (Paulin-Levasseur et al., 1989a). Therefore, this system can be exploited to examine the context of lamins A and C expression during differentiation.

In the present study, the HL-60 cell system was used to determine whether expression of lamins A and C was associated with the differentiated phenotype. HL-60 were treated with two different inducers of monocyte/macrophage differentiation: vitamin D_3 and TPA. A detailed kinetic analysis of lamins A and C protein expression throughout the differentiation period was made using immunofluorescence microscopy (IFM). The expression of vimentin, the monocyte marker 63D3 and transferrin receptor (TR) were assessed in like manner. HL-60 proliferation was monitored by cell counts, ^{3}H-thymidine incorporation and cell cycle analysis.

Induction of HL-60 cell differentiation by vitamin D_3 did not lead to expression of either lamins A/C or vimentin. Treatment with TPA, on the other hand, resulted in expression of these proteins in the vast majority of cells within 24 hours. Examination of marker expression and proliferation confirmed that both vitamin D_3 and TPA induced

differentiation in the treated cells. However, exposure to the vitamin resulted in cells which were monocyte-like, while TPA treatment gave rise to cells that more closely resembled macrophages.

Lamins A/C expression was clearly not associated with growth arrest in the vitamin D_3-treated cells, and was found to follow inhibition of replication in TPA-treated populations.

It was determined that lamins A/C expression was not coincident with expression of the differentiated phenotype. Vitamin-treated cells demonstrated dramatic increase in expression of 63D3, a late rise in TR expression and adherence of half the population to the substratum in the absence of lamins A and C. Kinetic analysis of TPA-treated cells demonstrated that lamins A/C expression followed dramatic morphological changes and adherence of the vast majority of the population to the substratum, as well as induction of vimentin expression. Lamins A/C expression was not coupled to any of these indicators of cell differentiation, including expression of the cytoplasmic IFP vimentin.

These results suggested that lamins A/C expression was a post-differentiation event in the HL-60 cell system. This was demonstrated to be the case for the murine peritoneal macrophages which served as a physiologically normal control cell type as well. Lamins A/C expression in these cells was not a function of lineage, but of physiological state.

Taken together, the results were consistent with the hypothesis of Röber and colleagues (1990b), who postulate that lamins A/C expression follows the loss of all developmental plasticity in the face of future environmental changes.

INTRODUCTION

THE CYTOSKELETON AND KARYOSKELETON

The cellular cytoskeleton and karyoskeleton are composed of lattices or networks of protein filaments. The cytoskeleton is involved in a multitude of general processes, such as cell division, maintenance of cell shape, cellular movement and cytoplasmic organization (Amos and Amos, 1991; Bray, 1992). It is also implicated in specialised functions such as the movement of cilia or flagella present on some cells. The karyoskeleton serves to maintain nuclear shape and is involved in the functional organization of the nucleus (Georgatos, 1994).

Three major classes of cytoskeletal fibres can be distinguished based on their diameter as seen with electron microscopy, their chemical constituents and their antigenic properties (Alberts et al., 1983; Amos and Amos, 1991; Bray 1992). The largest are the microtubules (25 nm in diameter), seemingly hollow cylinders made up of globular tubulin heterodimers. Intermediate filaments (10 nm diameter) may be composed of one or several of the family of intermediate filament proteins (IFPs), most of which are expressed in a tissue-specific pattern. Microfilaments (approximately 8 nm) are composed of globular actin subunits. Components of the karyoskeleton are less well characterised, with the exception of the lamina, a protein meshwork located at the inner face of the nuclear envelope. It is made up of lamins, a subfamily of intermediate filament proteins (for review see Georgatos, 1994).

Intermediate Filament Proteins

The intermediate filament proteins are encoded by a large multigene family. Six types of IFPs are currently recognised (for reviews see Robson, 1989; Albers and Fuchs, 1992).

Types I and II comprise the small acidic and larger basic keratin proteins respectively. In cells of epithelial origin, at least one pair of keratin proteins consisting of one type I and one type II are expressed and form heterodimers.

Type III includes a number of tissue-specific IFPs: desmin which is expressed in muscle cells; the glial fibrillary acidic proteins located in glial cells and astrocytes; peripherin, found in nervous cells and vimentin which is seen in cells of mesenchymal origin and in many cultured cell lines (Lazarides, 1982; Portier, Nechaud and Gros, 1984). All are known to form homopolymeric filaments in some cell types *in vivo*. In addition some may form heteropolymers (Lazarides, 1982).

Type IV IFPs are the neurofilaments, a subfamily of four proteins expressed in neurons, where they form heteropolymeric filaments.

Type V IFPs refer to the lamins, a subfamily in mammals of at least seven nuclear proteins. Unlike the other IFPs, lamins are found in a wide variety of cell types (Aebi et al., 1986; McKeon, Kirschner and Caput, 1986). This thesis will focus on this subfamily.

Finally, type VI IFP designates nestin, which forms filaments in the stem cells of the central nervous system (Lendahl, Zimmermann and McKay, 1990).

LAMINS

Lamin proteins have been studied extensively. Several lamin types have been characterised and their location within the cell is well known. The structure of the proteins has been explored and some insight gained into filament structure and dynamics.

A) Types

Mammalian lamins are classified as belonging to one of two groups. The A-type lamins include the major lamins A and C and the minor germ cell-specific lamin C_2 (Furukawa, Inagaki and Hotta, 1994). The B-type lamins include the major lamins B_1 and B_2 as well as several minor types, such as lamins D and E and the germ cell-specific lamin B_3 (Lehner et al., 1986b; Furukawa and Hotta, 1993). Most work to date has focussed on the major lamins A, B_1, B_2 and C. Their molecular weights differ slightly: lamin A is 70,000 daltons, the B lamins are approximately 67,000 daltons and lamin C is 60,000 daltons. They can also be distinguished by their isoelectric points: the B lamins are acidic (pI 5.7) while lamins A and C are near neutral (pI 6.4) (Shelton and Egle, 1979).

Lamin classification as intermediate filament proteins is based on sequencing (McKeon, Kirschner and Caput, 1986), structural and assembly characteristics (Aebi et al., 1986) and on cross-reactivity with an antibody that recognises an epitope common to most members of the IFP family (Gerace, Blum and Blobel, 1978).

The status of the lamins as a distinct IFP subfamily has been established through sequence analysis. All the lamins have extensive sequence homology, possessing positively

charged amino terminal domains and conserved motifs in the carboxyl terminal domain (Höger, Krohne and Franke, 1988). cDNA sequencing of lamins A and C revealed that they are identical over the length of the lamin C sequence, but that lamin A possesses additional sequences at the carboxyl terminus. They arise from alternate splicing of the transcripts of a single gene (McKeon, Kirschner and Caput, 1986; Lin and Worman, 1993). The B lamins arise from discrete gene sequences (Zewe et al., 1991).

B) Location

The lamins are transported to the nucleus quite rapidly after synthesis, where they are processed and polymerised (Gerace, Comeau and Benson, 1984). They form a highly insoluble, extraction-resistant mesh of filaments called the nuclear lamina (Dwyer and Blobel, 1976). The lamina is located at the interface of the inner nuclear envelope and the chromatin, and interacts directly or indirectly with both these components (Gerace, Blum and Blobel, 1978; Lebkowski and Laemmli, 1982; Gerace, Comeau and Benson, 1984; Ludérus et al., 1992).

Microscopic evidence has provided evidence of diversity of the lamina organization in different cell types. Electron micrographs of freeze-dried *Xenopus* oocyte nuclei revealed a regular, orthogonal mesh of fibres, each approximately 10 nm in diameter with average crossover spacings of 52 nm (Aebi et al., 1986). In contrast, processed three dimensional light microscopic images of the nuclei of *Drosophila* embryos and several cultured cell lines showed an irregular fibrillar network made up of larger fibrils and containing sizeable areas devoid of lamins (Paddy et al., 1990). It appears that the structure of the lamina varies. This diversity may reflect the differing composition of these lamina, or species and/or cell type variability.

Current evidence points to indirect interaction of the lamina with the nuclear envelope. Lamin B receptors have been identified in a number of species. The first were detected in chicken nuclear membrane (Worman et al., 1988; Worman et al., 1990) and in rat nuclear envelope (Senior and Gerace, 1988; Foisner and Gerace, 1993).

In vitro evidence suggests that lamins B_1, A and C are all capable of binding to chromatin. Southwestern blotting assays have demonstrated that lamin B_1 can bind matrix attachment regions of DNA, indicating a potential direct link for these elements (Ludérus et al., 1992). Binding of lamins A/C to metaphase chromosomes *in vitro* has been detected at the light microscope level and was specific to a chromosome surface element inasmuch as it was inhibited by conformational change of the chromosomes (Glass and Gerace, 1990).

C) Structure

The lamin proteins display a structure common to all intermediate filament proteins. The base of this structure is a central α-helical rod domain flanked by two variable, non-helical domains: the amino-terminal head and the carboxyl-terminal tail (Aebi et al., 1986, McKeon, Kirschner and Caput, 1986).

The central rod domain of the IFPs is the region most highly conserved between different members of this family. All have characteristic heptad repeat sequences in this region essential to generating its helical structure. The lamin rod domain can be separated into four α-helical subdomains (1A, 1B, 2A and 2B) separated by three short, non-helical linker sequences (L1, L12 and L2). The second of these subdomains, helix 1B, is longer in the lamins than in other vertebrate IFP and serves as a distinguishing characteristic of this subfamily (Steinert, Steven

and Roop, 1985). An epitope common to most intermediate filament proteins is found in the last 20 amino acid residues at the carboxyl end of the rod domain (Geisler et al., 1983). In addition, the lamins share conserved motifs in the 10 amino acid residues immediately flanking the two ends of the rod domain (Höger, Krohne and Franke, 1988).

The head and tail domains of all IFPs display less homology than the rod domain and considerable variation in size, particularly between members belonging to different types (Steinert, Steven and Roop, 1985). These flanking domains do not have a very regular secondary structure.

All the lamins have a short amino terminal head region. Their carboxyl termini vary in size. Lamins A and C, which are generated via alternate splicing of the transcripts of a single gene, differ only in their carboxyl termini (McKeon, Kirschner and Caput, 1986).

The tail domain of all three lamins features a nuclear localisation signal similar to that of the SV40 large T antigen. Mutation or deletion of this signal results in assembly of lamin polymers in the cytoplasm (Loewinger and McKeon, 1988).

A terminal motif similar to that found in ras proteins (CXXM) is present on prelamin A, the precursor form of this protein, and the B lamins (Fisher, Chaudhary and Blobel, 1986; McKeon, Kirschner and Caput, 1986). It serves in a manner analogous to the ras motif as the site of post-translational modifications (Clarke et al., 1988). In the case of the lamins, isoprenylation and carboxyl methylation of the cysteine residue of this motif have been identified (Kitten and Nigg, 1991). Isoprenylation makes the lamin tail domain hydrophobic and thereby assists or mediates non-specific insertion of these proteins in the nuclear envelope. This may be followed by receptor binding for the B lamins or incorporation into the lamina and loss of

the modified motif for lamin A (Holtz et al., 1989; Weber, Plessmann and Traub, 1989; Beck, Hosick and Sinensky, 1990; Kitten and Nigg, 1991).

D) Filament Structure

The organization of lamin proteins into lamina filaments has been partially characterised, as is true of intermediate filaments in general (for review see Dessev, 1992). Lamins are capable of heteropolymeric interactions both *in vitro* and *in vivo*. Binding assays have demonstrated heterotypic interactions of both lamins A and C with lamin B (Georgatos, Stournaras and Blobel, 1988). Incorporation of transfected lamin A into the lamina of cells which express only B-type lamins was seen by immunofluorescence microscopy (IFM). It occurred in a uniform manner over the entire nuclear rim, arguing for heterotypic association of the transfected protein with the native lamins (Horton, McMorrow and Burke, 1992). Filament formation involves lamin interaction on three levels: dimer formation, longitudinal association into polymers and lateral association into filaments.

The basic building block of lamin polymers is a dimer, whose formation has been classified as type I lamin interaction. In these dimers, parallel lamin monomers associate in register throughout their α-helical domains, forming a coiled coil. The amino- and carboxyl-terminal end domains project from both ends of the coiled regions.

Transfection of human lamin deletion mutants into Chinese hamster ovary cells, where their incorporation was monitored by human-specific antibodies, has indicated that the 10 amino acid residues at the carboxyl terminus of the rod domain are essential for lamin polymerisation (Loewinger and McKeon, 1988). The rod domain is well conserved in sequence and in structure

by all members of the intermediate filament family of proteins, thus they all assemble into dimers in a similar manner (Heins and Aebi, 1994).

Type II interactions involve the longitudinal association of lamin dimers in a head to tail fashion to form filamentous polymers. Electron microscopy studies of lamin B_2 and lamin A assembled *in vitro* showed that this association is mediated by the carboxyl- and/or amino-terminal end domains of the lamin proteins (Gieffers and Krohne, 1991; Heitlinger et al., 1991). A biochemical study demonstrating the decreased ability of lamins A and C terminal deletion mutants to form polymers confirmed this conclusion (Moir et al., 1991). In contrast to these longitudinal lamin associations, other intermediate filament proteins form side by side tetramers associated by their rod domains (Heins and Aebi, 1994).

Finally, type III lamin interactions consist of lateral associations of the elongated polymers in approximately half-staggered alignment to form thicker filamentous structures. This type of association also depends on interactions of the lamin end domains (Heitlinger et al., 1991).

E) Filament Dynamics

The nuclear lamina filaments are reversibly depolymerised during mitosis. Early evidence indicated that the B lamins remained attached to the remnants of the nuclear envelope while lamins A and C were solubilised during this transition (Gerace and Blobel, 1980). More recent work indicates that a portion of the B lamins are also solubilised (Chaudhary and Courvalin, 1993; Lourim and Krohne, 1993), although in some cases this appears to be true of only a minute fraction of these proteins (Meier and Georgatos, 1994). In addition to this mitotic

cycle, the lamina must also incorporate nascent lamin proteins during nuclear growth in interphase. The dynamics of lamina formation and disassembly have been examined in an effort to understand both the processes involved and the factors which control them.

The process of post-mitotic lamina assembly has been partially eludicated. The lamina of the daughter nuclei are made up of the existing lamin proteins which were dispersed during mitosis (Gerace, Blum and Blobel, 1978). Lamins which remained bound to the nuclear envelope remnant vesicles during mitosis (including the B lamins) would be returned to their interphase location via accumulation of the vesicles around the telophase chromosomes. Incorporation of soluble lamins (including mammalian lamins A and C) is a separate event in nuclear envelope formation both *in vitro* and *in vivo*. The bulk of lamins A and C incorporation follows fusion of the vesicularised envelope fragments and requires transport of the lamins through the pores of the daughter nuclei (Benavente, Scheer and Chaly, 1989; Newport, Wilson and Dunphy, 1990). It is not known whether lamina assembly proceeds from one or several organising regions, or involves random incorporation throughout the growing network.

Nascent lamin A incorporation into an existing lamina has been shown to occur in several discrete steps. A biochemical study of chicken embryo fibroblasts found that prelamin A was rapidly located to the soluble nuclear fraction upon synthesis (Lehner et al., 1986). A delay then occurred before lamin A was detected in the insoluble nuclear fraction, where it existed only as the shorter mature form. A microscopic study of the fate of native and microinjected biotinylated lamin A in 3T3 fibroblast cells also found very rapid transit of the lamins to the nucleus, where they accumulated in nucleoplasmic foci (Goldman et al., 1992). With time these foci became concentrated near the nuclear envelope, evolving into the typical rim staining pattern

seen for lamins. Taken together these results suggest that the rate limiting step in incorporation of nascent lamin A is processing to the mature form, followed by association with the nuclear envelope which appears to occur at a number of discrete loci at the nuclear rim.

Lamina disassembly involves release of lamins from the filamentous network. The lamins are believed to remain in a dimerized state throughout mitosis based on presence of dimers following *in vitro* disassembly of lamin polymers (Peter et al., 1991) and detection of both monomers and dimers in mitotic surf clam oocyte extracts of soluble proteins (Dessev et al., 1990). The control of lamina assembly/disassembly is better understood than that of any other intermediate filaments. The disassembly and reformation of the lamina which accompanies mitosis is known to be directly triggered by the level of phosphorylation of the lamin proteins (Gerace and Blobel, 1980; Gerace, Comeau and Benson, 1984).

Lamin phosphorylation by the protein kinase $p34^{cdc2}$-cyclin B initiates disassembly of the lamina of isolated nuclei (Peter et al., 1990; Dessev et al., 1991), of lamina filaments *in vitro* and of transfected chicken lamin B_2 protein filaments in temperature-sensitive cdc2 yeast mutants grown at the permissive temperature (Enoch et al., 1991). Disassembly-specific phosphorylation sites on mitotic or *in vitro* cdc2-treated lamins have been identified as two serine residues, one in the head domain and one in the carboxyl terminal domain, both close to their respective ends of the α-helical rod (Heald and McKeon, 1990; Ward and Kirschner, 1990; Peter et al., 1991). Phosphorylation of these sites affects head-to-tail interactions of the lamin proteins but not the rod-dependent dimer associations between them (Dessev et al., 1990; Heitlinger et al., 1991).

Dephosphorylation of these serine residues by an as-yet-unidentified phosphatase in telophase allows reformation of the lamina at the nuclear periphery in the daughter cells.

VARIABLE EXPRESSION OF LAMINS

In the early 1980's mammalian nuclear lamina was believed to uniformly consist of three lamin types: A, B and C. In the mid 1980's studies began to reveal a variability of the expression of certain lamin proteins both *in vivo* and *in vitro*.

A) Variable Expression in Vivo

The first indication of variability in lamin expression came from studies of *Xenopus* gametes and embryos. Studies of mouse embryos followed and demonstrated that variability of lamin expression was also present during mammalian embryogenesis.

Immunofluorescence microscopy (IFM) and immunoblotting experiments using lamin-specific antibodies demonstrated a changing pattern of lamin expression during *Xenopus* gametogenesis. Many types of lamins are recognised in this species. The expression of two types found in adult tissues, L_I and L_{II}, disappeared abruptly at pachytene in oocytes and upon differentiation of primary spermatocytes (Stick and Schwartz, 1983; Benavente and Krohne, 1985). Shortly thereafter novel lamin protein was detected in both gametes. The diplotene oocyte had a lamina made up of L_{III}, an A-type lamin, while spermatids and sperm had patchy lamina made up of lamin L_{IV} (Stick and Hausen, 1985; Benavente, Krohne and Franke 1985).

Turning to embryogenesis, these same groups both found that lamin L_{III} was the sole lamin detectable in embryos up to the mid blastula transition. After this point lamins L_I and L_{III}

were both present, joined by L_{II} after the mid gastrula stage. All three lamins were detected from this stage until late neurulation with the relative amount of lamin L_{III} declining steadily throughout this period. L_{III} then disappeared, and was seen again only after embryogenesis in the neural and muscle tissues of feeding tadpoles (Stick and Hausen, 1985; Benavente, Krohne and Franke, 1985).

Lamin expression has also been studied during early murine development. All lamin types (A, the B lamins and C) could be detected in mouse pronuclei and fertilised eggs by IFM and immunoblotting. However, lamins A and C staining disappeared during the first two to four embryonic cleavages and did not reappear in the embryo before 8 days gestation (of a total of 19 days). Lamin B was detected throughout this time and was concluded to be the only lamin expressed at the morula or blastula stages (Schatten et al., 1985; Stewart and Burke, 1987). An extensive immunocytochemical staining study of mouse embryos and newborn mice extended these findings (Röber, Weber and Osborn, 1989). Lamins A and C were undetectable throughout the first two thirds of mouse embryogenesis, which comprise the stages of germ layer formation and basic organogenesis. They reappeared in a tissue-variable pattern during the last third of gestation and the first 20 days of postnatal life. This time frame represents the stage of tissue differentiation. Myoblastic cells were the first to express lamins A and C, followed by epidermal cells. Several internal organs did not express the A type lamins until 5 to 20 days post partum. Lamin B was present in all cells at all stages studied.

B) Variable Expression in Vitro

The expression of lamins in various cell lines came under scrutiny following the first

report of lamin variation in mouse embryos. Results began to appear in 1987.

The first report of developmental variability of lamin expression in a cell culture system was made by Lebel and colleagues (1987). They examined the lamins present in mouse embryonic carcinoma cells induced to differentiate towards neuronal cells by retinoic acid. Lamin B was the only lamin present before the induction of differentiation, while lamins A and C appeared and increased over time with treatment. This expression of lamins A/C was shown to depend on *de novo* protein synthesis.

A survey of several cell types appeared almost simultaneously (Guilly et al., 1987). They found that HeLa cells and Epstein-Barr virus-transformed B lymphocyte cells (B-EBV cells) expressed all the lamins. In contrast, several human T lymphoblastic cell lines (KE-37 and T-IL-2 cells) were found to contain normal levels of lamin B, but express little or no lamins A and C. Northern blotting demonstrated that no mRNA for lamins A or C was detectable in these cells.

A study by Paulin-Levasseur and collaborators (1988) followed which compared the lamina composition of a number of myeloid- and lymphoid-derived cell lines characteristic of several stages of haematopoietic differentiation. Promyelocytic, myeloma and plasmacytoma cells were all represented. Each expressed only lamin B and lacked lamins A/C. Co-incidentally, all these cells also lacked vimentin, their expected cytoplasmic intermediate filament protein. No expression of alternate cytoplasmic IFPs was detected; the cells appeared to proliferate and survive without a network of intermediate filaments in their cytoplasm and with a lamina composed only of lamin B. A more detailed study was then undertaken of the HL-60 promyelocytic cell line. Following treatment with either DMSO to induce differentiation

towards granulocytes or TPA to induce the macrophage path, these cells were found to express both lamins A/C and vimentin. Northern blotting demonstrated that this new expression was controlled primarily at the transcriptional level (Paulin-Levasseur et al., 1989a).

Further studies focusing on the haematopoietic system confirmed the emerging trends. Cells representing several stages of lymphoid differentiation were surveyed (Guilly et al., 1990). Pre-B leukaemia cells, T lymphoblastic cell line KE 37 and rat thymocytes were found to express only lamin B. In contrast, peripheral blood T and B lymphocytes and cell lines RPMI-8866 and U266, representative of mature B cells, all expressed a full complement of lamins. Cells of the myelocytic lineage have also been studied by using primary cultures of peripheral blood monocytes and rat bone marrow grown under differentiation-promoting conditions (Röber et al., 1990). When isolated these cells were lamins A and C negative. After several days in culture they became lamins A/C positive concurrent with expression of accessory cell or macrophage morphology. This change in lamin expression was also seen during *in vivo* differentiation of cells of this lineage. Unstimulated peritoneal macrophages were lamins A and C negative, while five days after intraperitoneal thioglycollate stimulation over 80% of the cells expressed these proteins.

From these studies several conclusions have been reached. First, that lamin B is constitutively expressed. Following the identification of two B lamins in 1989 (Weber, Plessmann and Traub) it was shown that both are expressed in the same ubiquitous fashion (Höger et al., 1990). Secondly, that lamins A and C are usually co-expressed and that their expression in different tissues varies in a differentiation-dependent manner. This variation in expression appears to be transcriptionally controlled. However, it is known that co-expression

of lamins A and C is not absolute. A study of clones generated from the adrenal carcinoma cell line SW-13 revealed an exception which was found by IFM and immunoblotting to express substantial amounts of lamin C , but only trace quantities of lamin A (Paulin-Levasseur et al., 1989b).

LAMIN FUNCTIONS

In the early 1980's, when the mammalian nuclear lamina was thought to uniformly consist of all lamin types, lamins were thought to play a housekeeping role of providing a framework for chromatin organization (Gerace and Burke, 1988). This hypothesis was supported by electron microscopy studies of interphase DNA structure showing DNA loops attached to a protein scaffold. SDS-PAGE demonstrated that the scaffold was composed primarily of lamins (Hancock and Hughes, 1982). Further evidence was provided by microinjection experiments using antibodies which removed all the lamins in mitotic PtK_2 cells (Benavente and Krohne, 1986). This treatment resulted in daughter cells containing a nucleus-like structure which did not undergo normal decondensation of the chromatin and lacked nucleoli. Thus the presence of a lamina appeared to be a prerequisite to normal chromatin configuration and function. A possible role in giving structural support and tensile strength to the nucleus was also envisioned (Gerace and Burke, 1988).

The discovery of variability in lamin expression demanded a re-thinking of their functions. The view of lamins as housekeeping proteins needed to expand to accommodate the

existence of differentiation-induced lamin expression. Certain functions might be specific to the constitutive B lamins or to the variable A type lamins, while others could be characteristic of any lamin protein.

A) Functions of the B Lamins

Evidence has accumulated implicating the B lamins in several aspects of nuclear envelope and nucleoplasmic organization: in anchorage of the lamina to the nuclear envelope, post-mitotic nuclear envelope assembly, structural support for the growing nucleus and in the framework for functional chromatin organization.

It is thought that the B lamins serve to anchor the lamina to the inner nuclear envelope. This view is supported by the observations that they are more resistant to extraction than lamins A and C (Aebi et al., 1986) and that they are the only lamins to remain bound to the remnants of the nuclear envelope during mitosis (Gerace and Blobel, 1980). Evidence of nuclear envelope receptors for the B lamins has been found in several species, beginning with their identification in rat liver (Foisner and Gerace, 1988) and chicken (Worman et al., 1990).

As the mediator of lamina-nuclear envelope attachment, the B lamins are well positioned to play a role in reassembly of the nucleus after mitosis, by mediating aggregation of the envelope fragments with chromatin. To date, evidence for lamin involvement in nuclear envelope assembly has been conflicting.

An early *in vitro* study using isolated metaphase chromosomes (Burke and Gerace, 1986) demonstrated that immunodepletion of lamins in the test system inhibited the normal accumulation of nuclear envelope vesicles around the chromosomes. A more recent study

looked at nuclear reconstitution around sperm chromatin incubated in *Drosophila* embryo extracts (Ulitzer at al., 1992). This species expresses only one lamin protein, of which half is solubilised while half remains membrane bound during mitosis. Immunodepletion of lamins in the extract inhibited reconstitution of the nuclear envelope vesicles around the chromatin. Similar findings have gradually emerged from studies using a *Xenopus* oocyte extract-based system. Nuclear reconstitution studies using sperm DNA in normal and lamin-depleted *Xenopus* oocyte extracts originally found that vesicle association with chromatin and fusion to form a double membrane preceded active transport of lamin protein into the nucleus (Newport, Wilson and Dunphy, 1990). At this time, the system was thought to contain only the soluble A-type lamin L_{III}, and no vesicle-bound lamins. These studies did show that the association of vesicles with chromatin was mediated by unidentified proteins on both of these elements (Newport and Dunphy, 1992). Recent experiments have identified a vesicle-bound B-type lamin present in the *Xenopus* system in much smaller quantity than L_{III}. Reconstitution of nuclear envelope vesicles around chromatin does appear to involve this lamin (Lourim and Krohne, 1993). Finally, a recent confocal microscopy study of mitotic chicken hepatoma cells revealed that virtually all detectable lamin B co-localized with its receptor on nuclear envelope-derived vesicles throughout cell division (Meier and Georgatos, 1994). Co-assembly of these two elements around the decondensing chromatin as nuclear envelope reformation began was also recorded. Thus lamins seem to mediate nuclear envelope assembly in these systems by targeting vesicles to the chromatin.

In contrast stand the *in vivo* experiments of Benavente and Krohne (1986) previously described. They found that microinjection of lamin antibodies into mitotic PtK_2 cells resulted

in daughter cells which did form a nuclear envelope around their chromatin. However, the authors did not demonstrate that the antibody they were using reacted with all lamin types in mitotic cells of this lineage; antibody reactivity was checked only on interphase cell extracts, where there was limited recognition of lamin B. A recent biochemical and IFM study of mitotic HeLa cells provided further conflicting evidence. Chaudhary and Courvalin (1993) found that association of lamin B receptor-bound vesicles with the mitotic chromosomes appeared to precede clear association of lamin B-bound vesicles with the condensed chromatin. In addition they demonstrated that some lamin B is solubilised during mitosis, indicating mitotic release of at least some of this protein from its nuclear envelope receptor. Based on these results they conclude that the lamin B receptor, but not lamin B itself may mediate assembly of the nuclear envelope after mitosis.

The experiments outlined above have also provided evidence of lamina function in the domains of structural support and chromatin organization. After *in vitro* assembly of nuclei in lamin-free extracts, growth of the nuclear envelope was inhibited relative to controls. In addition, the assembled nuclei were very fragile (Newport, Wilson and Dunphy, 1990). These observations suggest that the lamina provides a physical framework to support the growing nucleus.

A role for the lamina in chromatin organization was suggested by the failure of lamin-free in vitro assembled nuclei to replicate their DNA (Newport, Wilson and Dunphy, 1990) and their atypical distribution of some proteins normally found in replicon clusters (Meier et al., 1991). These results are complimentary to *in vivo* experiments which showed that cells with nuclei depleted of some or all lamin types did not decondense their chromatin and did not form

nucleoli (Benavente and Krohne, 1986; Benavente, Scheer and Chaly, 1989; Benavente, 1991). A recent confocal microscopy study demonstrated the presence of nucleoplasmic lamin B foci in murine 3T3 cells during mid to late S phase (Moir, Montag-Lowry and Goldman, 1994). These foci were coincident with sites of DNA replication. Taken together, these results imply that the interaction of the lamin proteins with DNA has functional implications for replication and transcription.

While the housekeeping functions of providing nuclear strength and a framework for chromatin organization are implicated for the constitutively-expressed B lamins, it cannot be ruled out that lamins A and C are also capable of playing these roles when present. They may serve to modify or enhance the effects of the B lamins in these domains.

B) Lamins A and C Functions

Lamins A and C cannot be solely responsible for any fundamental cell housekeeping functions since they are absent in many cell types at early stages of differentiation. It has been proposed that they may exert genetic control at the 3-D level via chromatin binding. As such, the variable expression of lamins A and C would be part of the chain of genetic control of differentiation. Given that development involves both the switching on of tissue-specific genes and the turning off of others not necessary to the specialised cell, lamins A and C could act by binding to the chromatin and conveying on areas of DNA containing necessary genes a conformation accessible to the transcriptional apparatus and/or on those areas containing unnecessary genes a conformation which makes them inaccessible for transcription.

Lamins A/C binding to chromosomes has been repeatedly demonstrated *in vitro* (Burke

and Gerace, 1986; Burke, 1990; Glass and Gerace, 1990). This binding was shown to be specific to surface DNA sequences or proteins, as it could be disrupted by either trypsin digestion or change of chromosome conformation. Upon binding, the lamins became insoluble and formed a thin layer on the chromosome surface, changes comparable to the establishment of a polymerised lamina. The presence of this lamin coat rendered the chromosomes resistant to swelling in hypotonic conditions, suggesting that lamin binding locked the chromatin in a specific conformation (Glass and Gerace, 1990). The analogous role *in vivo* would be to establish a specific differentiation-related conformation of the chromatin. Recent evidence implies that change in chromatin conformation may arise following heterochromatin-lamin A interactions established during the nucleoplasmic processing of lamin A (Bridger et al., 1993). Using confocal microscopy of synchronised human fibroblast cells, the authors found small nucleoplasmic foci of lamin A present during early G_1 which were closely associated with areas of condensed chromatin. As the cells moved towards S phase the foci disappeared, presumably due to lamin A polymerisation.

EXPLORING LAMINS A AND C EXPRESSION IN VITRO

In order to form a more detailed picture of the initiation of lamins A and C expression in the context of cellular differentiation, cultured cells may be used as a model system. When cells capable of differentiation are exposed to an appropriate inducer, the appearance of lamins A and C can be tracked over the period of cell response to eludicate the time frame of their

expression. Markers of differentiation can be followed in parallel to lamins A/C detection to better understand the developmental context in which they are expressed. Such a model system is found in the HL-60 cell line.

A) The HL-60 Cell System

HL-60 cells were first cultured in 1977 from the peripheral blood of a woman suffering from acute promyelocytic leukaemia (Collins, Gallo and Gallagher, 1977). They have since become available for scientific research.

HL-60 have a promyelocytic cell morphology. They are small, round suspension cells with a very high nucleus/cytoplasm ratio. The nucleus typically features 2-4 prominent nucleoli. Cytoplasmic granules are frequently seen in the thin layer of cytoplasm surrounding the nucleus. Their doubling time is typically 24-30 hours but is often longer under traumatic conditions, such as immediately after culture manipulations.

HL-60 are commonly considered bipotential, capable of differentiation along either the granulocyte or monocyte/macrophage paths. However, they can also be induced to differentiate into eosinophils or basophils (Lübbert and Koeffler, 1988). In untreated cultures 5-15% of cells spontaneously express a mature phenotype, usually of granulocytes (Collins, Gallo and Gallagher, 1977; Paulin-Levasseur et al., 1989; Carson, unpublished results).

The multipotent nature of the HL-60 cell line makes it a particularly attractive model for studying differentiation-associated processes. Manipulation of the cells with different inducers can be exploited to distinguish between lineage-specific and general differentiation-associated processes by stimulating undifferentiated HL-60 commitment to distinct cell types. Inducer-

specific effects can be distinguished from differentiation-related changes by comparing the results of HL-60 treatment with several inducers of the same lineage.

B) HL-60 Differentiation

HL-60 can differentiate in response to a multitude of inducers. Among them, the tumour-promoting phorbol diester TPA (or PMA) and the principal hormonal form of vitamin D_3 *in vivo*, 1α,25-dihydroxycholecalciferol, engage the cells on the monocyte path and promote differentiation towards macrophages (Lübbert and Koeffler, 1988). This thesis will focus on HL-60 differentiation in response to these two inducers. HL-60 reaction to all inducers occurs in several stages: a lineage-independent precommitment phase precedes expression of the differentiated phenotype.

TPA was quickly identified as an inducer of HL-60 (Rovera, O'Brien and Diamond, 1979). Treatment with TPA gave rise to numerous markers of differentiation, including cessation of cell proliferation, accumulation of cells in the G_1 phase of the cell cycle, cell adherence and expression of a macrophage-like phenotype. Morphological changes included decrease in the nucleus/cytoplasm ratio, change in nuclear shape to reniform and decreased prominence of nucleoli and cytoplasmic granules. The cells became phagocytic and expressed several biochemical markers of the monocytic lineage (Rovera, Santoli and Damsky, 1979; Rovera, Olashaw and Meo, 1980).

TPA acts in part through activation of the protein kinase C pathway of intracellular phosphorylation (Castagna et al., 1982), although this alone has been shown to be insufficient to induce Hl-60 cell differentiation (Kreutter, Caldwell and Morin, 1985). In TPA-treated HL-

60, activation of the protein kinase C signalling pathway combines with other biochemical effects of the phorbol ester, including effects on Ca^{++} (Morin et al., 1987), to induce expression of differentiation-controlling and/or differentiation-specific genes.

Vitamin D_3 was identified as an HL-60 inducing agent a few years later (Miyaura et al., 1981). This compound also caused growth inhibition and cell cycle arrest in G_1 (Bar-Shavit et al., 1986). Cell adherence (in this case of 25-50% of cells) and phagocytic ability were induced. Once again, morphological alterations included a decrease in the nucleus/cytoplasm ratio and change in the nuclear shape to reniform. The pathway engaged by vitamin treatment was identified by detection of monocyte-specific cell surface antigens and enzyme activity in treated cells (Miyaura et al., 1985). Two-dimensional gel electrophoresis protein profiles showed that both TPA and vitamin D_3 treatment resulted in expression of some proteins specific to peripheral blood monocytes. While neither treatment resulted in a protein profile that mimicked the exact monocyte pattern, the vitamin D_3-exposed cells were closer than those induced with TPA (Murao et al., 1983).

Vitamin D_3 exerts its effects through interaction with a cytoplasmic receptor (Feldman et al., 1988). Vitamin binding causes a conformational change in the receptor, translocation of the vitamin-receptor complex to the nucleus, DNA binding by the zinc-finger domain of the receptor and initiation of transcription (Haussler et al., 1988). During induction of HL-60, the vitamin must initiate the expression of differentiation-controlling and/or differentiation-associated genes.

By studying the timing of HL-60 response to inducers and the metabolic cascades they trigger, the group led by Andrew Yen at the University of Iowa has developed a model of HL-

60 differentiation as a multistage process that is only partially lineage specific.

Confirming previous observations (Rovera, Olashaw and Meo, 1980), Yen and Chiao (1983) found that HL-60 cells are responsive to inducers for a period of time during S phase. If exposed to a differentiation signal during this window of opportunity the cells will progress into a precommitment stage. This stage is not lineage specific (Yen et al., 1987). It involves changes in gene regulation and programs the cell for growth arrest if differentiation proceeds further within a few generations (Yen, Powers and Fishbaugh, 1986).

If the cells continue to be exposed to the same inducer, or are re-exposed to the same or an alternate inducer within their labile memory period, they will respond by expression of the differentiated phenotype appropriate for the compound which is present. This expression will occur quickly in cases of inducer re-exposure, apparently without the time usually taken for precommitment programming (Yen et al., 1987). During this differentiation stage, expression of lineage-specific antigens and biochemical markers can be detected.

C) Lamins A and C Expression During HL-60 Differentiation

A recent report from the lab of Dr. Micheline Paulin-Levasseur describes a study of lamins A/C and vimentin expression in HL-60 cells induced to differentiate towards macrophages by the phorbol ester TPA (Paulin-Levasseur and Julien, 1992).

Using IFM and immunoblotting, it was determined that lamins A and C expression followed cessation of growth, cell adherence and visible changes in cell size, nucleus/cytoplasm ratio and number of nucleoli. In contrast, vimentin was expressed maximally as cell adherence reached its peak and before complete expression of the differentiated phenotype. Thus, lamins

A and C expression is a very late event during TPA-induced differentiation. As such, it may serve to lock in place the developmentally specific pattern of gene expression once it has been established.

Several aspects of TPA-induced differentiation may be limiting factors in such a study. Firstly, the time course is very short; all events take place within 48 hours. This could make it a challenge to see the sequence of events clearly. Secondly, TPA is a cytotoxic agent. A more physiological, less harmful inducer would provide a test system in which the risk of cell death events being confused with cell differentiation events was much lower.

THESIS OBJECTIVE AND APPROACH

The principal objective of this research was to determine whether lamins A and C protein expression was associated with the differentiated phenotype, and to further define the context of lamins A/C expression in the pattern of differentiation events in hopes of gaining insight into their function(s). To this end, the HL-60 cell system was employed, using TPA and vitamin D_3 to induce differentiation along the monocyte/macrophage path.

The kinetics of lamins A and C expression during differentiation was monitored using immunofluorescent staining with a monoclonal antibody to lamins A/C. The kinetics of vimentin expression was simultaneously followed by staining with a polyclonal antibody to vimentin. Since vimentin is the expected cytoplasmic IFP in these cells and is also expressed only after differentiation (Paulin-Levasseur et al., 1988), a comparison between lamins A/C and this

protein appeared potentially informative. Lamins A/C and vimentin expression was also monitored in mouse peritoneal macrophages to provide a differentiated, normal cell control with which to compare the HL-60 results.

Population increase, inhibition of replication and expression of cell surface antigens were all monitored throughout treatment in order to place the expression of lamins A/C into the context of cellular differentiation.

Population growth was assessed by haemocytometer counts of trypan blue dye-excluding cells. Inhibition of replication was monitored by measuring ^{3}H-thymidine incorporation and by flow cytometer analysis of propidium iodide-stained cells.

Expression of two cell surface markers that change with differentiation was assessed by IFM. Transferrin receptors (TRs) are present on immature cells of the promyelocytic lineage, are not expressed on monocytes and are detectable on some tissue-specific macrophages or those generated *in vitro* via culture of monocytes (Andreesen et al., 1986). Thus they were expected to be present in untreated HL-60, decline initially in response to inducer and reappear concurrent with the mature macrophage phenotype. The presence of TR was monitored by staining with the monoclonal antibody L5.1. The monoclonal antibody 63D3 recognises a cell surface protein of approximately 200 kD present on monocytes and a small fraction of granulocytes. It does not stain untreated HL-60 cells or macrophages (Ugolini et al., 1980; Nunez et al., 1982). When used to stain induced HL-60 cells, the frequency of 63D3-positive cells was expected to increase with time of treatment, then decrease as the cells became more macrophage-like. Expression of these markers in mouse macrophages was also assessed.

In summary, the time course of lamins A and C expression relative to the appearance of

a number of markers of cell differentiation was followed in hopes of generating a more precise picture of the developmental context of lamins A/C expression and thus to gather clues as to their functions.

CULTURE OF HL-60 AND MACROPHAGES

A) HL-60 Cell Culture

HL-60 cells were obtained from the American Type Culture Collection (Rockville, MD, No. CCL240). Cells were maintained as asynchronous suspension cultures in Dulbecco's modified Eagle medium (DMEM) (GIBCO Canada Inc., Burlington, Ontario), supplemented with 10% fetal calf serum (GIBCO Canada) and 1% antibiotic-antimycotic (GIBCO Canada). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2.

To induce differentiation, 1,25-dihyroxyvitamin D_3 (BIOMOL Research Laboratories, Plymouth Meeting, PA) in an ethanolic solution was added to growing cultures with densities of 2.5 - 2.7 x 10^5 cells/ml, to produce a final vitamin concentration of 5 x 10^{-7} M and a final ethanol concentration of 0.05 μl/ml. Controls received the same concentration of ethanol alone. Beginning at 48 hours, cultures were supplemented daily with 20% volume of fresh media which contained either 5 x 10^{-7}M vitamin D_3 (test cultures) or 0.05 μl/ml ethanol alone (controls). Inductions were carried out over 120 or 240 hours.

TPA (Sigma Chemical Co., St. Louis, MO) -stimulated differentiation was induced by adding the drug in an ethanolic solution to growing cultures, as for vitamin D_3 treatment. The final drug concentration was 3 x 10^{-8}M and the final ethanol concentration in test and control

cultures was 0.1 μl/ml. Inductions were carried out for 48 hours.

In one case, induction with TPA and vitamin D_3 was carried out in parallel on cells from a single starting population. Results of this experiment were consistent with those of individual inductions.

B) Primary Culture of Intraperitoneal Macrophages

Primary culture of macrophages was carried out as described (Edelson and Cohn, 1976). Production of intraperitoneal macrophages was stimulated in male Balb-c mice (Jackson Laboratories, Bar Harbour, ME) by injection of 0.5 ml pristane (Aldrich Chemical Co., Milwaukee, WI). Seven days later, intraperitoneal cells were harvested by lavage with sterile culture medium and concentrated by centrifugation. Any red blood cells present were opsonized by a 5 minute incubation in 0.83% ammonium chloride (BDH Inc., Toronto, Ontario). The remaining cells were cultured in RPMI-1640 (GIBCO Canada) supplemented with 20% fetal calf serum and 1% antibiotic-antimycotic at a density of 5 x 10^5 cells/ml. Cells were maintained at 37°C in a humid atmosphere of 5% CO_2. After 60 minutes, nonadherent cells and debris were removed by washing with several changes of fresh medium. Adherent macrophages were maintained in culture for up to 7 days.

ASSESSMENT OF CELL PROLIFERATION

During induction of differentiation by vitamin D_3 and TPA, cell growth was tracked by

population counts, measurement of ^{3}H-thymidine incorporation and flow cytometric analysis of the cell cycle distribution of propidium iodide-stained cells.

A) Assessment of Cell Density

Viable cells were counted at each sampling point on a Neubaur haemocytometer. Viable cells were distinguished by their ability to exclude 0.1% trypan blue dye in physiological saline solution. At each time point, four counts were made for each sample and the cell density calculated using the mean. Adjustment for any dilutions made in the vitamin D_3- treated cultures was then calculated by multiplying the cell density observed by 120% for each dilution performed. Cell density was monitored in a minimum of three independent experiments with each inducer. The mean cell densities for each sample, along with standard error, were plotted using Sigmaplot 5.1 (Jandel Scientific, San Raphael, CA), a software program written for this purpose.

B) Assessment of ^{3}H-Thymidine Incorporation into DNA

Incorporation of ^{3}H-thymidine was measured as previously described (Paulin-Levasseur and Julien, 1992) in triplicate 1 ml aliquots of control and test cells at each sample point. Aliquots were incubated with 2μCi/ml of ^{3}H-thymidine (DuPont Canada Inc., Mississauga, Ontario) for 90 minutes at 37°C. Samples were then frozen at -20°C until harvesting. To harvest DNA, samples were thawed and collected onto glass microfiber filters (Whatman International Ltd., Maidstone, England). Sample containers were washed twice with ice cold saline (9g/l NaCl), and the wash added to the glass filters. A third saline wash of the filters was

followed by two washes in ice cold 5% trichloroacetic acid (BDH Inc.). The acid-insoluble precipitate which remained was washed with ice cold 90% ethanol and the filters were placed in vials to which 4 ml of cytoscint cocktail (ICN Biomedicals Inc., St. Laurent, Quebec) was added. Radioactivity was measured on a Packard 2000CA liquid scintillation counter, which tracked the CPM over a three minute period for each sample. The mean count from the triplicate samples was used to calculate the CPM/10^6 cells. If the value of one of the triplicates diverged from the other two by more than 25% it was discarded and the mean of the remaining two values was used for the calculation. Three independent experiments with each inducer were performed, and the mean CPM/10^6 cells value for each sample, along with the standard error, were plotted using Sigmaplot 5.1.

C) Assessment of Cell Cycle Distribution

Propidium iodide staining of DNA was performed at each sample point on control and treated samples, using the DNA-Prep reagent kit (Coulter Electronics of Canada, Inc., Burlington, Ontario) in accordance with manufacturer's instructions. 100μl aliquots of cells were mixed well with equal quantities of lysis buffer, followed by addition of 1 ml of propidium iodide solution to each sample. Samples were vortexed and then incubated for 15-30 minutes at room temperature. Stained cells were analyzed on a Coulter Profile II flow cytometer. DNA content was quantified by measuring the amount of propidium iodide-based red fluorescence present in each cell. Raw data was recorded as a histogram of the distribution of DNA content in the sample population. Cell cycle analysis of the histograms was performed using Multicycle (Phoenix Flow Systems, San Diego, CA), a software program written for this purpose. The

percentage of cells in G_1, S and G_2/M were recorded for each sample. All samples fit the cell cycle model well enough to produce chi square values of less than 0.4. Cell cycle distribution was monitored in three independent experiments with each inducer, and the mean percentage of cells in each phase of the cycle was plotted for each sample, along with the standard error, using Sigmaplot 5.1.

ASSESSMENT OF PROTEIN EXPRESSION

Throughout the course of differentiation in response to vitamin D_3 or TPA, the expression of lamins A and C, vimentin, transferrin receptor and the monocyte-specific 63D3 antigen were monitored at the protein level by indirect immunofluorescence staining.

A) Indirect Immunofluorescence Staining for Lamins A/C and Vimentin

Control and nonadherent treated cells were harvested by centrifugation and resuspended in cell culture medium at a density of approximately 1 x 10^6 cells/ml. Cells were allowed to settle onto coverslips which had been coated with poly-l-lysine (Sigma Chemical Co.) during 10 minutes incubation in a humid chamber. Adherent cells were collected on coverslips placed in the tissue culture dishes at the start of the induction.

Indirect immunofluorescence microscopy was carried out as previously described (Chaly et al., 1984). Coverslips were washed twice for 30 seconds each time in PBS, then fixed for 5 minutes in 3% paraformaldehyde (J.B. EM Services Inc., Dorval, Quebec). Free aldehyde

groups were reduced by three 4 minute incubations in 0.1% sodium borohydride (BDH Inc.) in PBS. Cells were permeabilized by a 20 minute wash in 0.2% Triton-X-100 (BDH Inc.) in PBS and then rinsed three times for four minutes in PBS. Primary antibody incubation was then carried out for one hour in a humid chamber with antibodies diluted in PBS, followed by three four minute washes in PBS. Coverslips were returned to the humid chamber for a 45 minute incubation with secondary antibodies diluted in PBS, then given a final set of three four minute washes in PBS. Finally, cellular DNA was counterstained for 30 seconds in 0.1% Hoechst 33258 (Sigma Chemical Co.) in PBS and the coverslips were mounted on slides in p-phenylene diamine-glycerol mounting medium.

Lamins A and C were detected using the Burke mouse monoclonal IgM antibody (generously supplied by Peter Traub, Max-Planck Institut fur Zellbiologie, Ladenburg bei Heidelberg, Deutschland) at a dilution of 1 in 10. This antibody recognises both lamins A and C, but not lamin B by immunofluorescence (Paulin-Levasseur et al., 1988). Lamin staining was visualised with FITC-conjugated goat α-mouse IgM(μ) (Mandel Scientific Co., Guelph, Ontario), used at a dilution of 1 in 125. Vimentin was detected using a goat polyclonal antibody described previously (Giese and Traub, 1986). This antibody was also the gift of Peter Traub, and was used at a dilution of 1 in 1500. Vimentin staining was visualized by FITC-conjugated rabbit α-goat IgG (H&L) (Cappel, through Organon Teknika, Scarborough, Ontario), diluted 1 in 250.

B) Indirect Immunofluorescence Staining for Transferrin Receptor and Monocyte Specific Antigen

Cells were harvested as described for lamins A/C and vimentin staining, and indirect

immunofluorescence staining carried out as described by Rudnicki and McBurney (1987). Coverslips were given two 30 second washes in a 1:1 mixture of PBS/DMEM, then incubated with undiluted primary antibody for 10 minutes in a humid chamber. Cells were then given two 30 second washes in PBS/DMEM, and incubated with the secondary antibody diluted in PBS for 45 minutes in the humid chamber. Two more 30 second washes in PBS/DMEM followed and then cells were fixed for 15 minutes at -20°C in fresh methanol/acetic acid (95:5 v/v). Finally, fixed cells were air dried briefly and rehydrated by three 5 minute washes in PBS. Coverslips were mounted in p-phenylene diamine mounting medium as for lamins A/C and vimentin staining.

Transferrin receptors were identified by the mouse monoclonal IgG present in L5.1 cell supernatant, while monocyte marker protein was detected by the mouse monoclonal IgG present in 63D3 cell supernatant. Both hybridomas were obtained from the American Type Culture Collection. FITC-conjugated goat α-mouse Ig (IgA, IgG, IgM) H&L (Cappel) was used to visualize both primaries, at a dilution of 1 in 150.

C) Immunofluorescence Microscopy Assessment of Protein Expression

Stained cells were observed on a Zeiss Axiophot microscope equipped with epifluorescence and phase optics, and photographed using Ilford XP2-400 film.

For treated and control cells at each sample point, four hundred cells on each of duplicate slides were observed and scored as positive or negative for antibody staining. The percentage of positive cells in the populations was calculated using the mean of the duplicate slide values. Counts were made in at least three independent experiments for each inducer, and the mean

percent positive cells, along with the standard error were plotted for each sample using Sigmaplot 5.1.

RESULTS

HL-60 CELL MORPHOLOGY DURING DIFFERENTIATION

Changes in HL-60 cell morphology in response to treatment with either 5 x 10^{-7}M vitamin D_3 or 3 x 10^{-8}M TPA were detected by phase contrast microscopy as described in Materials and Methods. No changes were noticed in response to treatment with ethanol alone. Treatment with both differentiation inducers caused a fraction of the population to become adherent. Vitamin D_3-treated cells displayed only subtle changes in morphology, while dramatic alterations in TPA-treated cells were recorded. The morphology of isolated mouse peritoneal macrophages was found to resemble most closely the TPA-treated cells which became adherent.

Untreated HL-60 cells are depicted in figures 1A and 2A. They are small, round suspension cells with limited cytoplasm. Most feature one or two prominent nucleoli. Some display a few cytoplasmic granules.

Both inducers of differentiation used for this study were administered in an ethanol solution. Figures 1C and 2C depict control cells given the same final concentration of ethanol as the inducer-treated cells. They served to demonstrate any effects generated by the presence of the alcohol rather than the inducer. As the cells in these micrographs demonstrate, ethanol-treated controls resembled untreated HL-60 cells.

Figures 1E, 1G, 2E and 2G depict vitamin D_3-treated HL-60 cells after ten days in the

Figure 1. Detection of lamins A and C proteins in HL-60 cells and mouse peritoneal macrophages. Lamins A and C expression was detected using immunofluorescent staining with a mouse monoclonal anti-lamins A/C antibody, visualised with FITC-conjugated goat anti-mouse IgM antibody. Typical results for untreated HL-60 (A and B), control HL-60 treated with ethanol alone for 24 hours (C and D), HL-60 treated with vitamin D_3 for 240 hours (E,F,G and H), HL-60 treated with TPA for 24 hours (I,J,K and L) and peritoneal macrophages kept in primary culture for 24 (M and N) or 168 hours (O and P) are shown. Results for those HL-60 which remained in suspension after treatment (E,F,I and J) and those which became adherent (G,H,K and L) are both included. Phase contrast (A,C,E,G,I,K,M,and O) and corresponding fluorescent images (B,D,F,H,J,L,N and P) are shown.

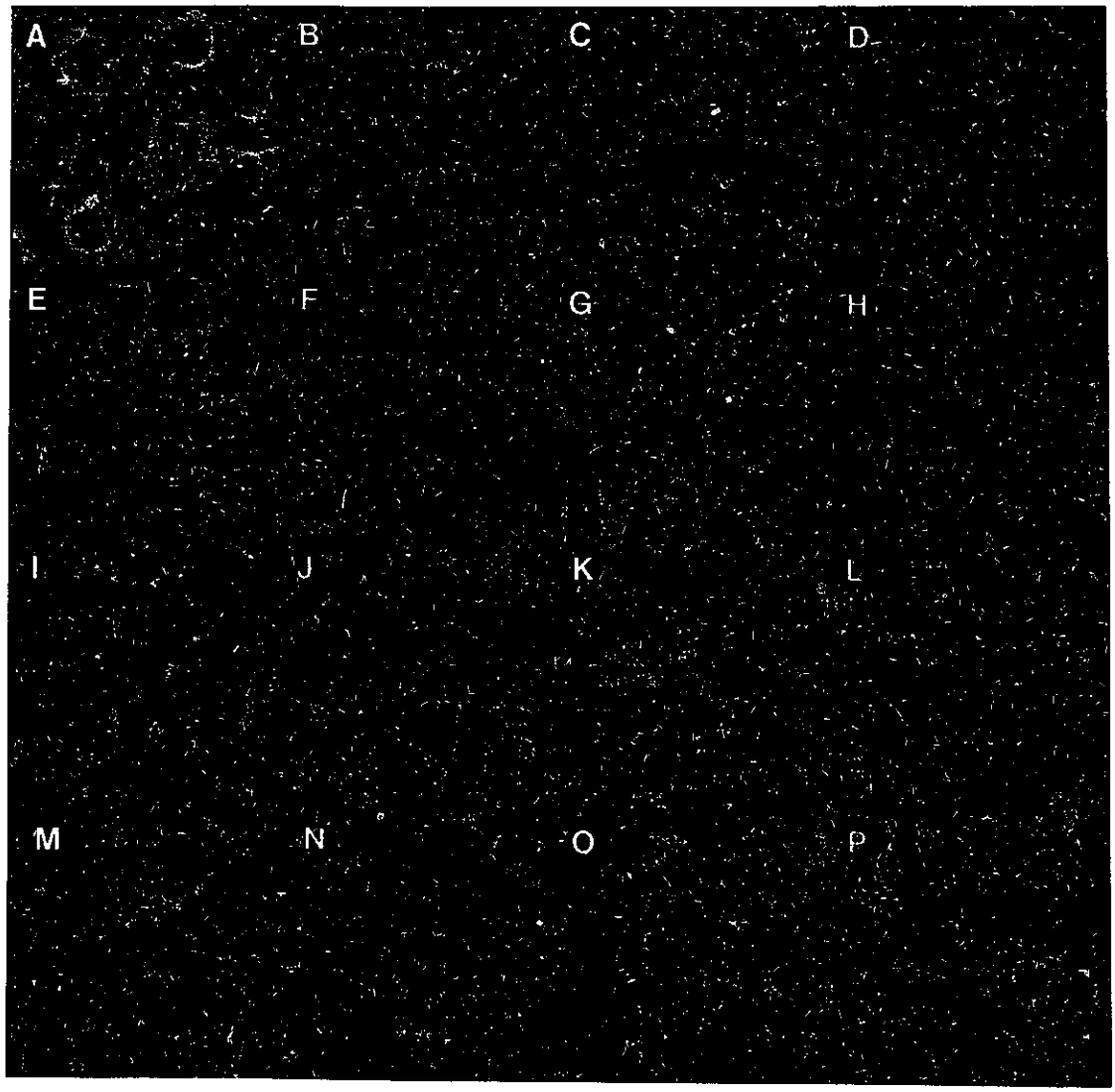
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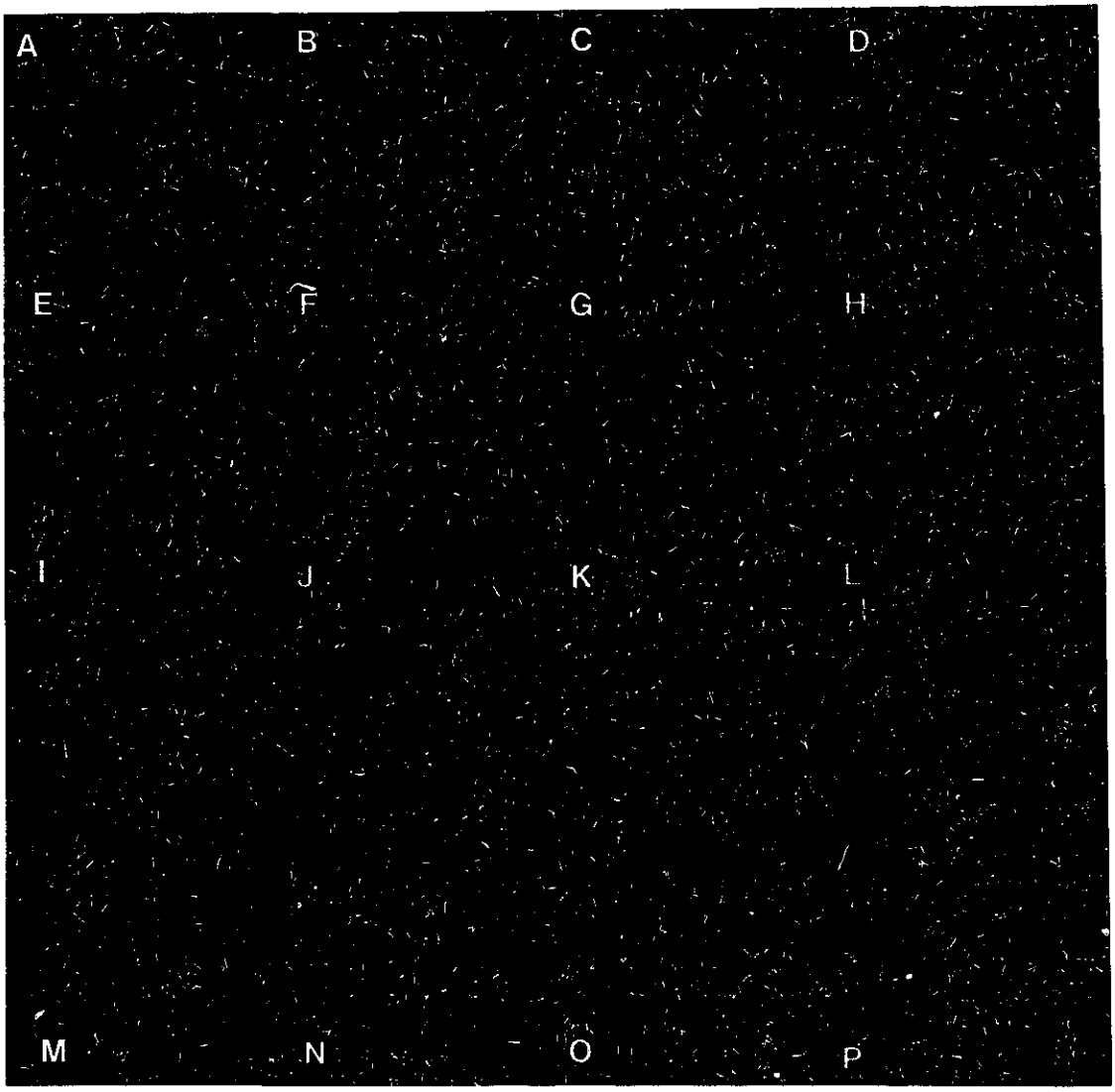
Figure 2. Detection of vimentin protein in HL-60 cells and mouse peritoneal macrophages. Vimentin was detected using immunofluorescent staining with a goat polyclonal anti-vimentin antibody, visualised with FITC-conjugated rabbit anti-goat IgG antibody. Typical results for untreated HL-60 (A and B), control HL-60 treated with ethanol alone for 24 hours (C and D), HL-60 treated with vitamin D_3 for 240 hours (E,F,G and H), HL-60 treated with TPA for 24 hours (I,J,K and L) and peritoneal macrophages kept in primary culture for 24 (M and N) or 168 hours (O and P) are shown. Results for those HL-60 which remained in suspension after treatment (E,F,I and J) and those which became adherent (G,H,K and L) are both included. Phase contrast (A,C,E,G,I,K,M,and O) and corresponding fluorescent images (B,D,F,H,J,L,N and P) are shown.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presence of the inducer. Those which remained in suspension throughout treatment are shown in figures 1E and 2E, while figures 1G and 2G depict cells which became adherent. The adherent subpopulation typically represented 20 - 50% of the vitamin-treated cells. All the cells remained small and round, although the adherent cells had a slight decrease in their nucleus/cytoplasm ratio. Nucleoli were typically less prominent than in untreated HL-60 (figures 1E, 2E and 2G) and some of the cells had lobed nuclei (figure 2G, lower cell). An increase in cytoplasmic granules was often seen in both subpopulations (figure 1E, lower cell, 1G, both cells and 2E, cell on right).

Cells treated for 24 hours with 3 x 10^{-8}M TPA displayed dramatic morphological changes. Figures 1I and 2I show TPA-treated cells which remained in suspension while figures 1K and 2K depict those which became adherent. The nonadherent cells were typically round with limited cytoplasm, but larger than untreated HL-60. Nuclei were often an elongated or a lobed shape (figure 2I) and nucleoli less prominent than in controls. Approximately 80% of the TPA-treated cells became adherent and underwent dramatic changes in their morphology. They became large, elongated, motile cells with abundant cytoplasm containing a profusion of granules. Nuclei were elongated and lobed (figure 1K, lower cell) or oval-shaped with prominent nucleoli (figures 1K, upper cell and 2K).

In order to provide a normal cell type for comparison to the induced HL-60 cell populations, mouse peritoneal macrophages were isolated. They were examined after either 24 hours (figures 1M and 2M) or 168 hours in culture (figures 1O and 2O). The size and morphology of the macrophage depicted in figure 1M is strikingly similar to that of the adherent TPA-treated cells seen in figures 1K and 2K. The macrophages had nuclei which were

elongated (figure 1M) or oval with prominent nucleoli (figure 1O, 2M and 2O). They often contained an abundance of prominent cytoplasmic granules. In both these aspects of their morphology they closely resembled the TPA-induced adherent subpopulation of cells. Figures 1O, 2M and 2O depict the common morphology of the macrophages in culture which may represent those cells which were not actively motile at the time of fixation.

LAMINS A AND C EXPRESSION DURING DIFFERENTIATION OF HL-60 CELLS

A) Detection of Lamins A and C Expression by Immunofluorescence Microscopy

Lamins A and C expression during induction of differentiation in HL-60 cells by either 5 x 10^{-7}M vitamin D_3 or 3 x 10^{-8}M TPA was detected by epifluorescence microscopy after indirect immunofluorescent staining performed as described in Materials and Methods. Examples of the various lamins A/C staining patterns seen are shown in Figure 1.

Untreated HL-60 cells were predominantly lamins A/C-negative, with only 5-10% of the population expressing lamins A and C. Figure 1A depicts a typical group of untreated HL-60 cells which, as figure 1B demonstrates, were lamins A/C-negative. During the course of thesis research, construction of D'Iorio Hall adjacent to Gendron, where the tissue culture room was housed, disturbed ideal experimental conditions for prolonged periods of time. During these periods up to 25% of the untreated HL-60 cell population expressed lamins A and C, although all experimental trends observed remained consistent. In order to minimize the error introduced into the data by these fluctuations in the background rate of lamins A/C expression, results are

presented as the percent positive cells seen above or below the level of positive cells in the untreated HL-60 population.

As the cell in figures 1C and 1D demonstrates, ethanol-treated controls typically did not express lamins A and C.

In HL-60 cells induced to differentiate over a period of ten days with 5 x 10^{-7}M vitamin D_3 only a small minority of cells expressed lamins A and C. Figures 1E-1H were chosen to include examples of these positive cells. Figures 1E and 1F depict vitamin-treated cells which remained in suspension. In figure 1F, lamins A/C staining is seen around the periphery of the lobed nucleus in the bottom right hand corner. An example of vitamin-induced cells which became adherent is seen in figures 1G and 1H. Clearly, the top nucleus in figure 1H is lamins A/C-positive. Both these examples are quite faint. This reflects reality; when present, lamins A/C staining in these cells was usually not very bright.

Figures 1I-1L provide examples of lamins A/C expression in cells treated for 24 hours with 3 x 10^{-8}M TPA. Figures 1I and 1J show nonadherent cells, while 1K and 1L depict cells in the adherent subpopulation. Virtually all TPA-treated cells expressed lamins A and C. The intensity of lamins A and C staining in these cells varied markedly among the population, as seen in figure 1J. On the whole, lamins A/C staining was brighter in positive TPA-treated cells than in positive vitamin D_3-treated cells.

Finally, figures 1M-1P depict lamins A and C expression in mouse peritoneal macrophages cultured for either 24 (figures 1M and 1N) or 168 hours (figures 1O and 1P). The macrophages were predominantly lamins A/C-negative shortly after isolation, but expressed the proteins after several days in culture. On the whole, the lamins A/C-positive macrophages

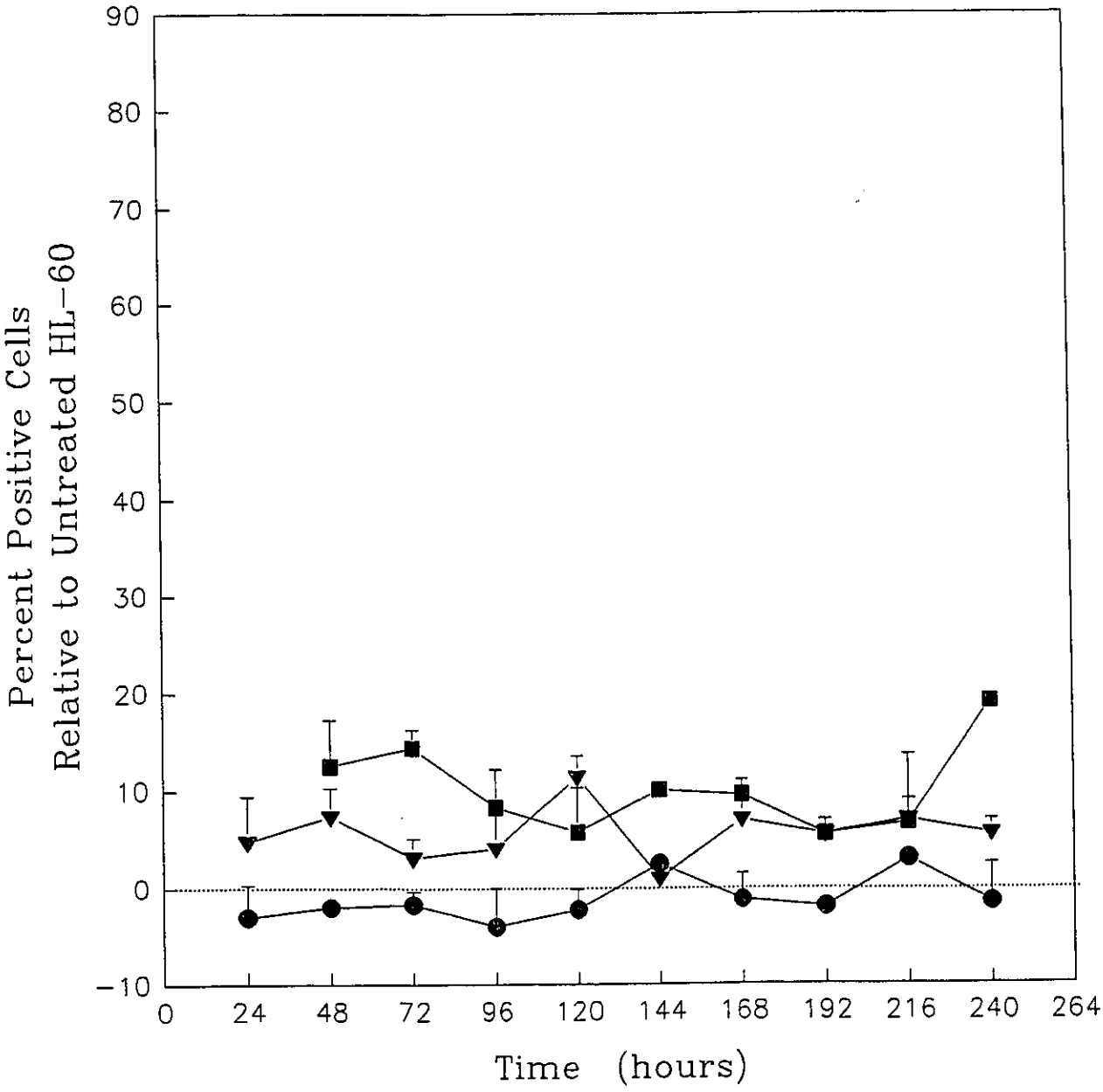
stained quite brightly, as demonstrated in figure 1P.

B) Kinetic Analysis of Lamins A and C Expression

Lamins A and C expression throughout the course of differentiation was assessed at the light microscope level via population counts, as described in Materials and Methods. At regular time intervals over the period of induction, counts were made of untreated HL-60 cells, ethanol-treated controls, treated cells which remained in suspension and treated cells which became adherent.

The kinetics of lamins A and C expression in response to induction of differentiation by 5×10^{-7}M vitamin D_3 is depicted in figure 3. The dashed line at the 0 mark indicates the level of lamins A/C expression in untreated HL-60 cells. The expression of these proteins in ethanol treated controls clearly does not differ significantly from this baseline. Both vitamin D_3-treated populations had levels of lamins A and C expression approximately 5 - 10% above the level of the controls. However, this apparent difference was not significant at most time points, as indicated by the error bars. T-tests (performed by the Sigmaplot 5.1 software program) comparing the treated populations with the ethanol control population at the individual time points showed significant differences at the $P > 0.05$ level for the adherent cell values at 72 and 96 hours only. T-tests comparing the nonadherent and adherent subpopulations of treated cells at the individual time points showed that they differed significantly from each other at the $P > 0.05$ level at 72 hours and 240 hours only. The error bars at these time points confirm these few differences. More importantly, the level of lamins A and C expression in treated cells did not change throughout the induction period; no overall increasing or decreasing trend is evident

Figure 3. Kinetic analysis of lamins A and C expression in HL-60 cells during induction of differentiation by 5 x 10^{-7} M vitamin D_3. Cells expressing lamins A and C as detected by immunofluorescent staining were quantified as a percentage of the total cell population. Results are expressed as the percentage of positive cells in treated cultures relative to those in untreated HL-60 control cultures. The dashed line indicates the zero point established by the level of protein expression in these control cultures. Results are the average of triplicate experiments. Error bars indicate standard error of the multiple measurements. Results for ethanol-treated control cultures are indicated by the filled circles (●), those for vitamin D_3-treated cells which remained nonadherent by filled triangles (▼) and those for vitamin-treated cells which became adherent during the course of treatment by filled squares (■).


Percent Positive Cells
Relative to Untreated HL-60
90
80
70
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50
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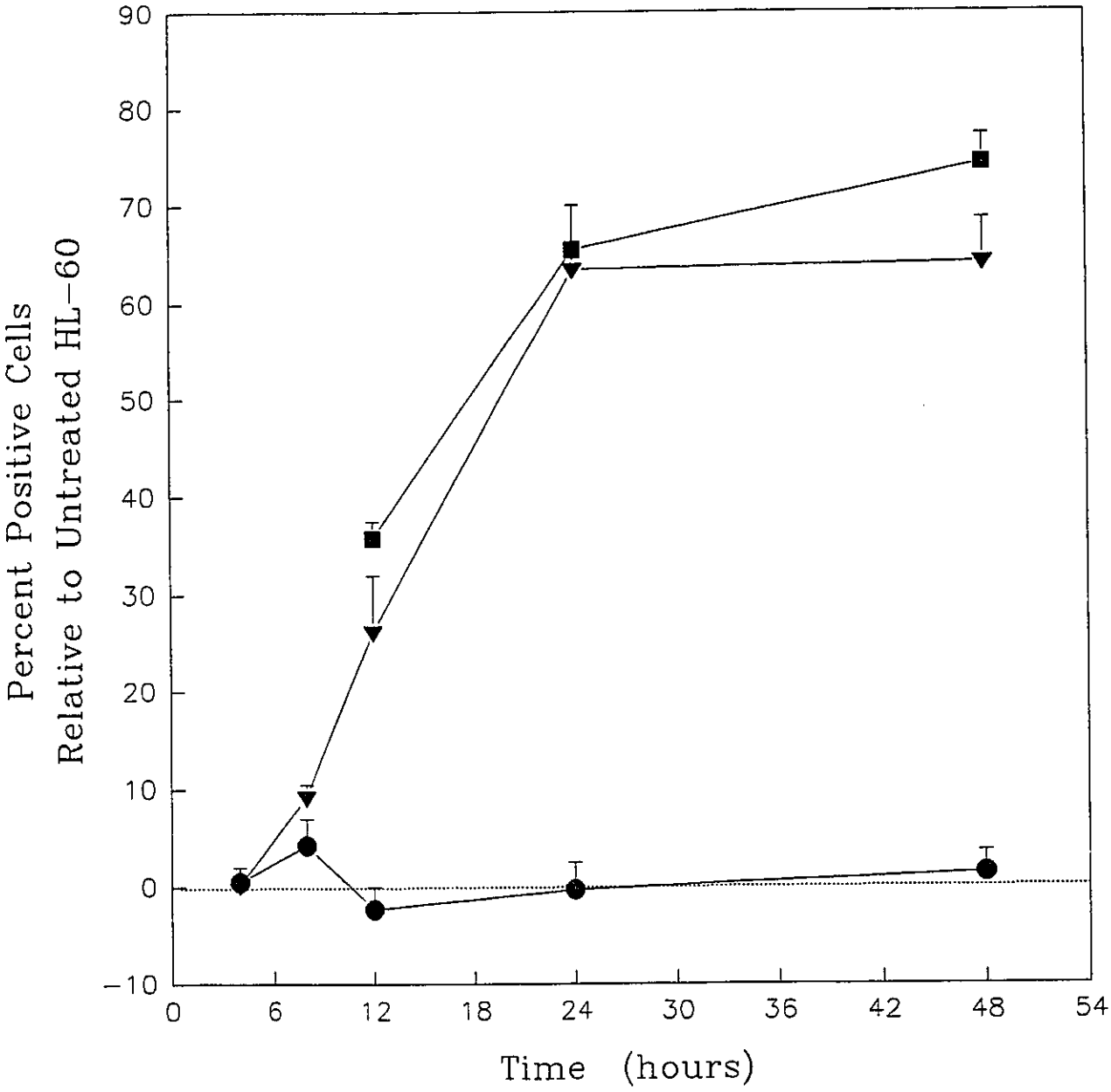
192
216
240
264
Time (hours)

in the graph. Induction of differentiation with vitamin D_3 did not promote increased expression of lamins A and C proteins in HL-60 cells.

Figure 4 depicts the kinetics of lamins A and C expression in HL-60 cells in response to induction of differentiation by 3 x 10^{-8}M TPA. As in figure 3, the dashed line indicates the level of expression in untreated HL-60 cells. Once again lamins A and C expression in ethanol-treated controls showed no differences from that in untreated HL-60 cells. In dramatic contrast to the results obtained with vitamin D_3 treatment, both TPA-induced subpopulations showed a marked increase in the expression of lamins A and C proteins. All treated cells began to show significant increase in lamins A/C expression relative to controls after 12 hours of treatment, as seen by the error bars on the graph and confirmed by t-tests comparing the treated cells to ethanol controls at this time point. The sharply rising, linear and almost parallel slopes of their plots on the graph indicate that the increase in lamins A/C expression occurred at a rapid and uniform rate from 8 hours through to 24 hours of treatment in both subpopulations. By 24 hours, there was an increase in lamins A/C expression of approximately 65% over control levels in both treated subpopulations. This level of expression remained steady in the nonadherent population through to 48 hours of treatment, while in the adherent population a small increase to approximately 75% above control level was seen. This slight difference in the two treated populations was significant, as seen by the error bars on the plot and confirmed by a t-test comparing the two treatment values at the 48 hour time point. Figure 4 clearly demonstrates that the induction of differentiation of HL-60 cells with TPA promotes expression of lamins A and C proteins by the vast majority of the treated cells within 24 hours of treatment.

Lamins A and C expression in mouse peritoneal macrophages was also examined in order

Figure 4. Kinetic analysis of lamins A and C expression in HL-60 cells during induction of differentiation by 3 x 10^{-8} M TPA. Cells expressing lamins A and C as detected by immunofluorescent staining were quantified as a percentage of the total cell population. Results are expressed as the percent positive cells in treated cultures relative to those in untreated HL-60 control cultures. The dashed line indicates the zero point established by the protein expression level in these control cultures. Results are the average of triplicate experiments. Error bars indicate standard error of the multiple measurements. Results for ethanol-treated control cultures are indicated by the filled circles, those for TPA-treated cells which remained nonadherent by filled triangles and those for TPA-treated cells which became adherent by filled squares.


Percent Positive Cells
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Time (hours)

to provide a normal, mature cell type for comparison with the cells generated after differentiation of HL-60 by the two inducers. Macrophages were stained for lamins A and C at two time points; shortly after isolation (24 hours) and after one week in culture (168 hours). These time points were chosen to represent the inactivated and activated form of macrophages, respectively. The results of all immunofluorescent stainings of macrophages performed for this thesis are contained in Table 1. Only 22% of macrophages were lamins A/C positive at 24 hours, while 91% of the population were expressing these proteins at 168 hours. Thus, the expression of lamins A and C proteins in macrophages appears to be related to their functional state rather than their cell type.

VIMENTIN EXPRESSION DURING DIFFERENTIATION OF HL-60 CELLS

A) Detection of Vimentin Expression by Immunofluorescence Microscopy

The expression of the IFP vimentin in HL-60 cells as they underwent differentiation in response to vitamin D_3 or TPA treatment was examined at the light microscope level using immunofluorescent staining. This protein is the expected tissue-specific cytoplasmic IFP type in these cells. It was thought that a comparison of lamins A/C expression with vimentin expression throughout the induction of the HL-60 cells would allow interpretation of the significance of the lamins A and C results in the broader context of IFP expression during differentiation. Examples of vimentin staining in HL-60 cells before and after induction of differentiation can be seen in Figure 2.

Table 1. Expression of lamins A and C, vimentin, monocyte marker 63D3 and transferrin receptor in primary cultures of mouse peritoneal macrophages. Cells expressing these antigens were quantified as a percentage of the total cell population. Results are the average of triplicate experiments.

Time (hours)	Percent Positive Cells			
	Lamins A/C	Vimentin	63D3 (Monocyte Marker)	L5.1 (Transferrin Receptor)
24	22 ± 2.5 %	100 %	13 ± 3.5 %	78 ± 2 %
168	91 ± 1 %	100 %	24 ± 1.5 %	79 ± 4.5 %

In untreated HL-60 populations (figures 2A and 2B) and in ethanol treated control populations (figures 2C and 2D), cells were predominantly vimentin-negative. A minority of 5-10% of cells in control cultures grown under good tissue culture conditions expressed vimentin protein. As seen with lamins A and C expression in control populations, the background level of vimentin expression increased in the less than ideal tissue culture conditions experienced throughout part of the thesis research, reaching levels of up to 25% positive cells.

Examples of vitamin D_3-induced HL-60 cells stained for vimentin after 10 days of treatment can be seen in figures 2E-2H. Figures 2E and 2F depict cells which remained in suspension throughout treatment, while figures 2G and 2H show cells which became attached to the substrate. In both cases, micrographs which showed both positive and negative cells in the population were selected. The vimentin-positive cell seen in 2F has very few filaments, most of which are concentrated in a small juxtanuclear "cap". A few extend into the cytoplasm at one side of the nucleus. The positive cell seen in figure 2H has a more extensive network of filaments. They too are concentrated on one side of the nucleus, but radiate throughout the cytoplasm in that area.

Vimentin expression in TPA-induced HL-60 cells which remained nonadherent after 24 hours is depicted in figures 2I and 2J, while examples of treated cells which became adherent by this time point are seen in figures 2K and 2L. The nonadherent cells show extensive networks of vimentin throughout their cytoplasm, with a concentration of fibres on one side of the nucleus. Vimentin filaments are present throughout the large cytoplasmic extensions of the cell seen in figure 2L. The juxtanuclear concentration of fibres seen in the previous examples is less marked here.

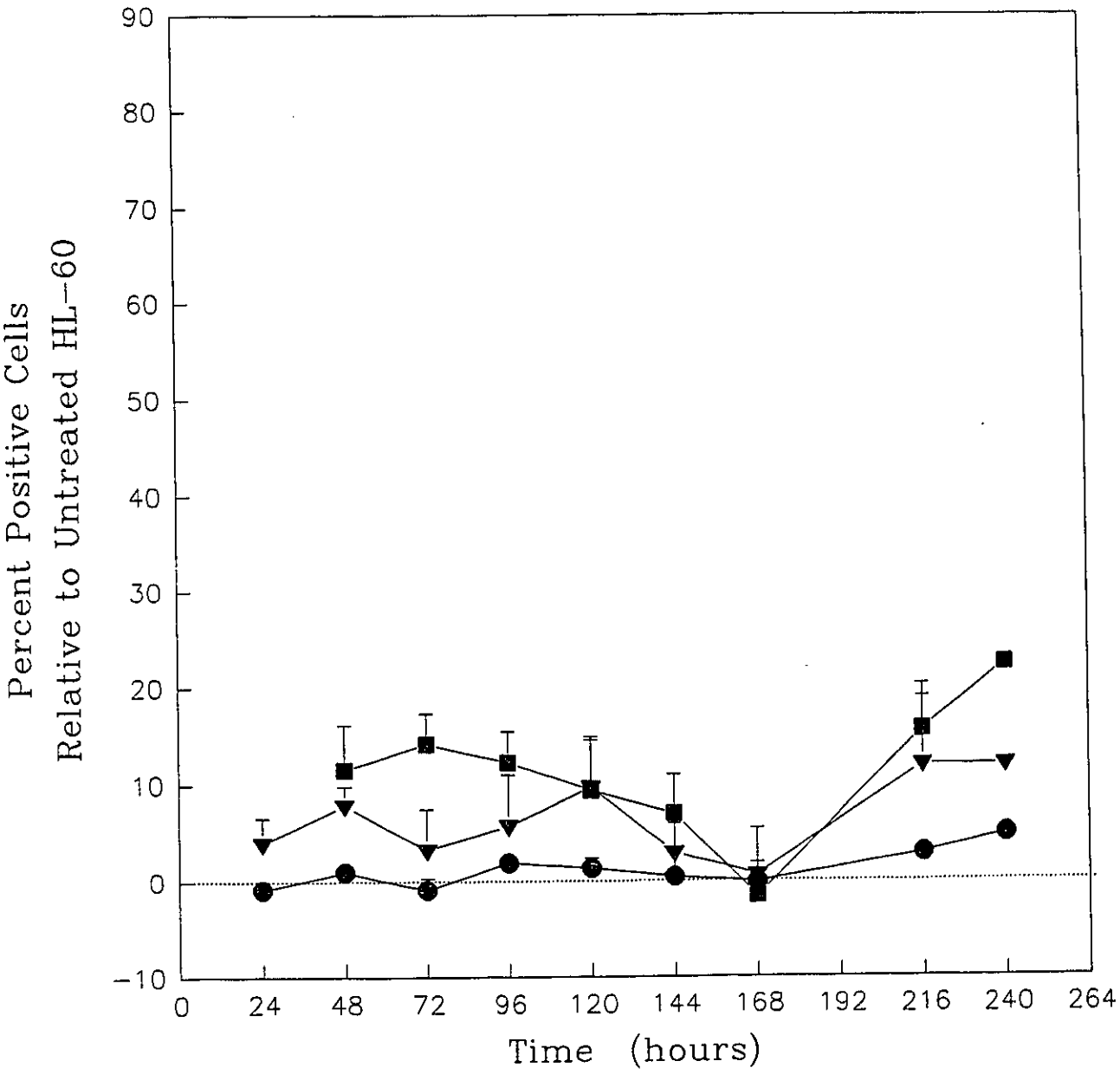
Vimentin expression was also examined in primary cultures of mouse peritoneal macrophages in order to provide a normal cell type for comparison with the results of the HL-60 differentiation experiments. Figures 2M and 2N depict a macrophage cultured for 24 hours, while figures 2O and 2P dépict a macrophage at 168 hours in culture. The cells seen in both of these figures have an abundant network of vimentin filaments which radiate throughout their cytoplasm, showing no obvious juxtanuclear concentration.

B) Kinetic Analysis of Vimentin Expression

The kinetics of vimentin expression in response to vitamin D_3 or TPA treatment was assessed by cell counts in the same manner as lamins A/C expression. The results are presented as percent positive cells above or below the level of expression seen in the untreated HL-60 control populations.

A kinetic analysis of vimentin expression in HL-60 cells induced to differentiate by 5 x 10^{-7} M vitamin D_3 is shown in figure 5. The ethanol-treated control population had levels of vimentin expression similar to those in untreated HL-60 cells throughout the entire period of differentiation. Vimentin expression in the vitamin-treated subpopulation which remained in suspension appeared to be about 5 - 10% higher than that seen in the ethanol controls. As indicated by the error bars on the two plots and confirmed by t-tests comparing the individual data points, this difference was not significant at the $P>0.05$ level. The treated cells which became adherent showed slightly higher levels of vimentin expression than the nonadherent cells up until 120 hours of treatment and again at the 240 hour time point, where they rose to 20% above the level seen in ethanol controls. T-tests of the individual data points indicated what the

Figure 5. Kinetic analysis of vimentin expression in HL-60 cells during induction of differentiation by 5 x 10^{-7} M vitamin D_3. Cells expressing vimentin as detected by immunofluorescent staining were quantified as a percentage of the total cell population. Results are expressed as the percentage of positive cells in treated cultures relative to those in untreated HL-60 control cultures. The dashed line indicates the zero point established by the level of protein expression in these control cultures. Results are the average of triplicate experiments. Error bars indicate standard error of the multiple measurements. Filled circles indicate results for ethanol-treated controls, filled triangles those of vitamin-treated cells which remained nonadherent and filled squares the results for vitamin-treated cells which became adherent.


Percent Positive Cells
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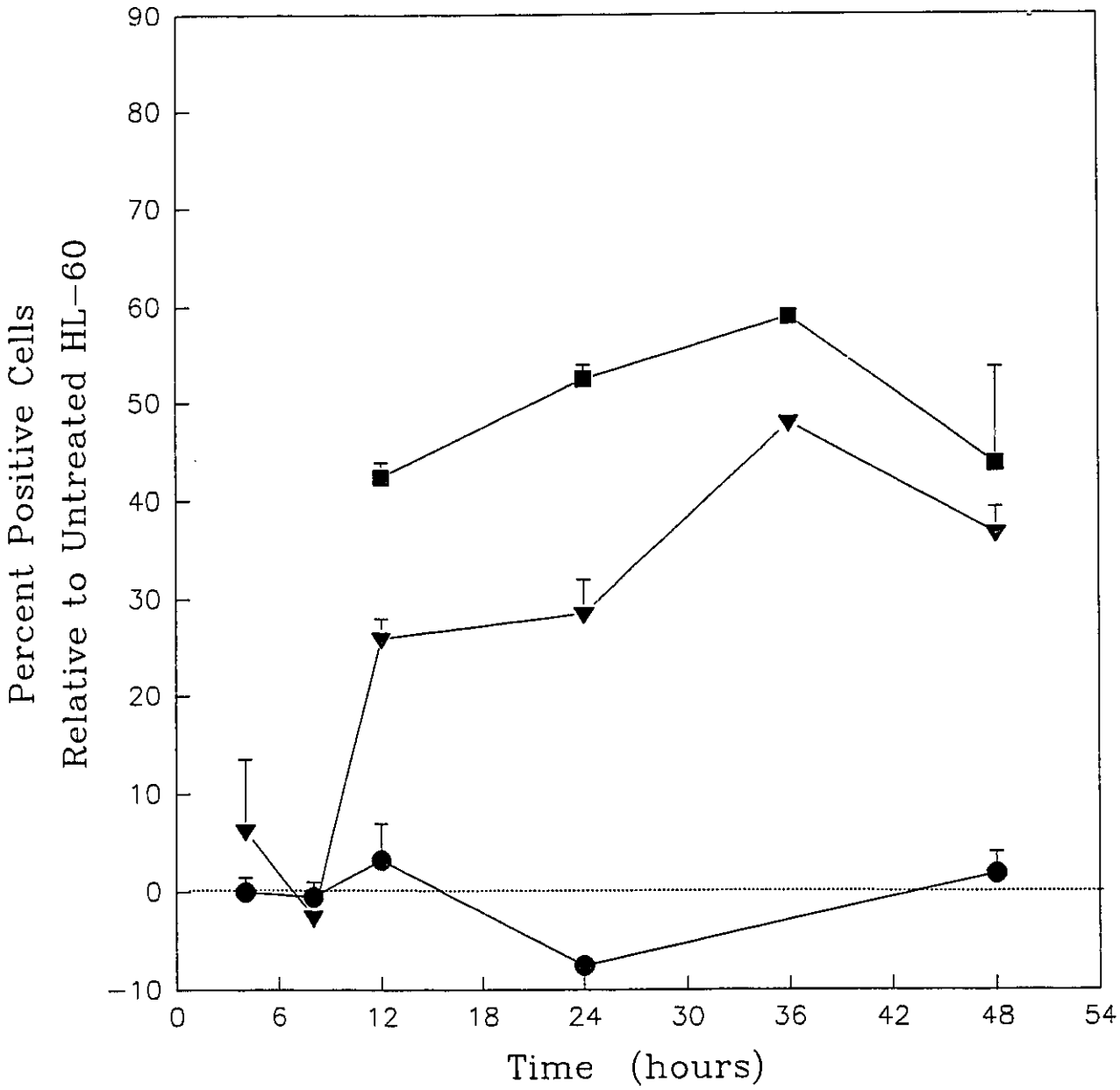
216
240
264
Time (hours)

error bars on the plot demonstrate: the only significant differences from control values occurred at the 72 hour and 240 hour time points. Clearly the trend observed in both treated populations did not differ significantly from the pattern of vimentin expression in control populations, with the possible exception of the final time point. It is possible that a slight but significant rise in expression of vimentin occurred in the treated populations after 216 hours of exposure to the inducer. However, this increase was seen in only 10 - 20 % of the treated cells. The vast majority remained vimentin-negative. As was found for lamins A and C expression, differentiation of HL-60 cells in response to vitamin D_3 treatment did not result in the expression of vimentin.

A kinetic analysis of vimentin expression throughout TPA-induced differentiation of HL-60 is presented in figure 6. Vimentin expression in ethanol-treated control populations did not vary significantly from that seen in untreated HL-60 cells during the period of induction. In contrast, both the nonadherent and adherent TPA-treated subpopulations showed a dramatic rise in the proportion of cells expressing vimentin protein. No significant effect of TPA induction on vimentin expression was seen before the 8 hour time point, but by 12 hours of exposure to the drug the level of vimentin expression in both subpopulations of treated cells had increased significantly. It continued to rise until 36 hours of treatment. Vimentin expression declined from 36 to 48 hours, but still remained significantly higher than the control populations. Vimentin expression in the adherent subpopulation was 10 - 20% higher than in the treated cells which remained in suspension throughout the 12 hour to 48 hour time points. As indicated by the error bars on the graph and confirmed by t-tests of the individual data points, this difference was significant for all time points except 48 hours. Despite this small quantitative difference between

Figure 6. Kinetic analysis of vimentin expression in HL-60 cells during induction of differentiation by 3 x 10^{-8} M TPA. Cells expressing vimentin as detected by immunofluorescent staining were quantified as a percentage of the total cell population. Results are expressed as the percentage of positive cells in treated cultures relative to those in untreated HL-60 control cultures. The dashed line indicates the zero point established by the level of protein expression in these control cultures. Results are the average of triplicate experiments. Error bars indicate standard error of the multiple measurements. Filled circles indicate results for ethanol-treated controls, filled triangles those of TPA-treated cells which remained nonadherent and filled squares the results for TPA-treated cells which became adherent.


Percent Positive Cells
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Time (hours)

the two TPA-treated subpopulations, it is clear that the trends of change in vimentin expression are the same for both of them. The rise in expression of vimentin from 12 to 36 hours occurred at a similar rate for both subpopulations, as seen by the nearly parallel slopes of the plots. The decline from 36 to 48 also occurred at similar rates. Echoing the results of kinetic analysis of lamins A and C expression in TPA-treated cells, vimentin expression was found to increase significantly compared to control populations by 12 hours of induction. However, it increased for the first 36 hours of treatment only, declining somewhat by the final time point. Vimentin expression was efficiently induced by TPA treatment during the middle events of the response process and at a lesser level during the latest stages.

As documented in Table 1, essentially all murine peritoneal macrophages in primary culture were vimentin-positive at both the time points studied. Expression of vimentin appears to be characteristic of the macrophage cell type.

MONOCYTE/MACROPHAGE MARKER EXPRESSION DURING DIFFERENTIATION OF HL-60 CELLS

To assess whether treated cells were indeed differentiating along the monocyte/macrophage path, the expression of several proteins characteristic of these cell types was monitored throughout HL-60 induction by vitamin D_3 or TPA. One of these was the monocyte-specific cell surface protein identified by the monoclonal antibody 63D3. This protein is considered an early marker of the monocyte lineage and is expressed throughout monocyte

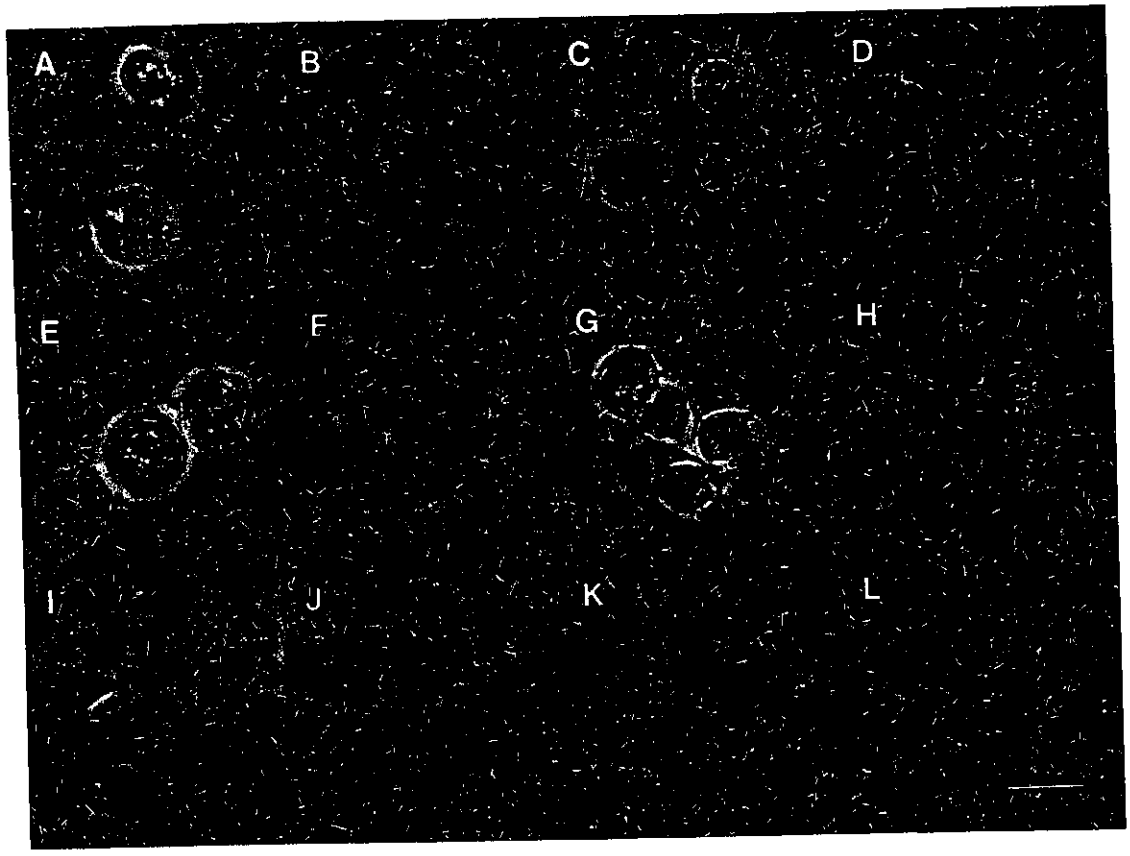
maturation (Ugolini et al.,1980; Nunez et al., 1982). The other marker studied was TR protein, another cell surface antigen. It was identified by the monoclonal antibody L5.1. TR is present on undifferentiated HL-60 cells, although the level of its expression in this population is known to vary with the proliferative state of the culture. It is absent in monocytes, but is expressed in macrophages (Andreesen et al., 1986). Thus, it was expected that L5.1 staining would disappear as the treated cells engaged the monocyte path and reappear when and if they became more macrophage-like.

A) Microscopic Detection and Kinetic Analysis of Monocyte Marker 63D3 Expression

The expression of monocyte marker protein during HL-60 cell differentiation was detected using indirect immunofluorescent staining with the 63D3 antibody as described in Materials and Methods. The kinetics of 63D3 antigen expression during induction of differentiation in HL-60 by 5 x 10^{-7} M vitamin D_3 or 3 x 10^{-8} M TPA was assessed by cell counts. The expression of this marker in mouse peritoneal macrophages in primary culture was detected and quantified in like manner.

Examples of the 63D3 staining patterns seen in the treated and control HL-60 populations studied for this thesis are found in figure 7. Figures 7A and 7B depict untreated HL-60 cells. They were largely 63D3 negative, as were the ethanol-treated controls pictured in figures 7C and 7D. Less than 10% of these populations expressed the 63D3 antigen under good tissue culture conditions. Figures 7E-7H give examples of 63D3 staining in vitamin D_3-treated cells at the 240 hour time point, both those which remained in suspension (7E and 7F) and those which became adherent (7G and 7H). These subpopulations were predominantly 63D3 positive.

Figure 7. Detection of monocyte marker 63D3 protein in HL-60 cells and mouse peritoneal macrophages. 63D3 expression was detected using a mouse monoclonal anti- 63D3 antibody, visualised with an FITC-conjugated goat anti-mouse IgG antibody. Typical results for untreated HL-60 (A and B), control HL-60 treated with ethanol alone for 24 hours (C and D), HL-60 treated with vitamin D_3 for 240 hours (E,F,G and H), HL-60 treated with TPA for 24 hours (I and J) and peritoneal macrophages kept in primary culture for 168 hours (K and L) are shown. Results for those HL-60 which remained in suspension after treatment (E and F) and those which became adherent (G,H,I and J) are both included. Phase contrast (A,C,E,G,I and K) and corresponding fluorescent images (B,D,F,H,J and L) are shown.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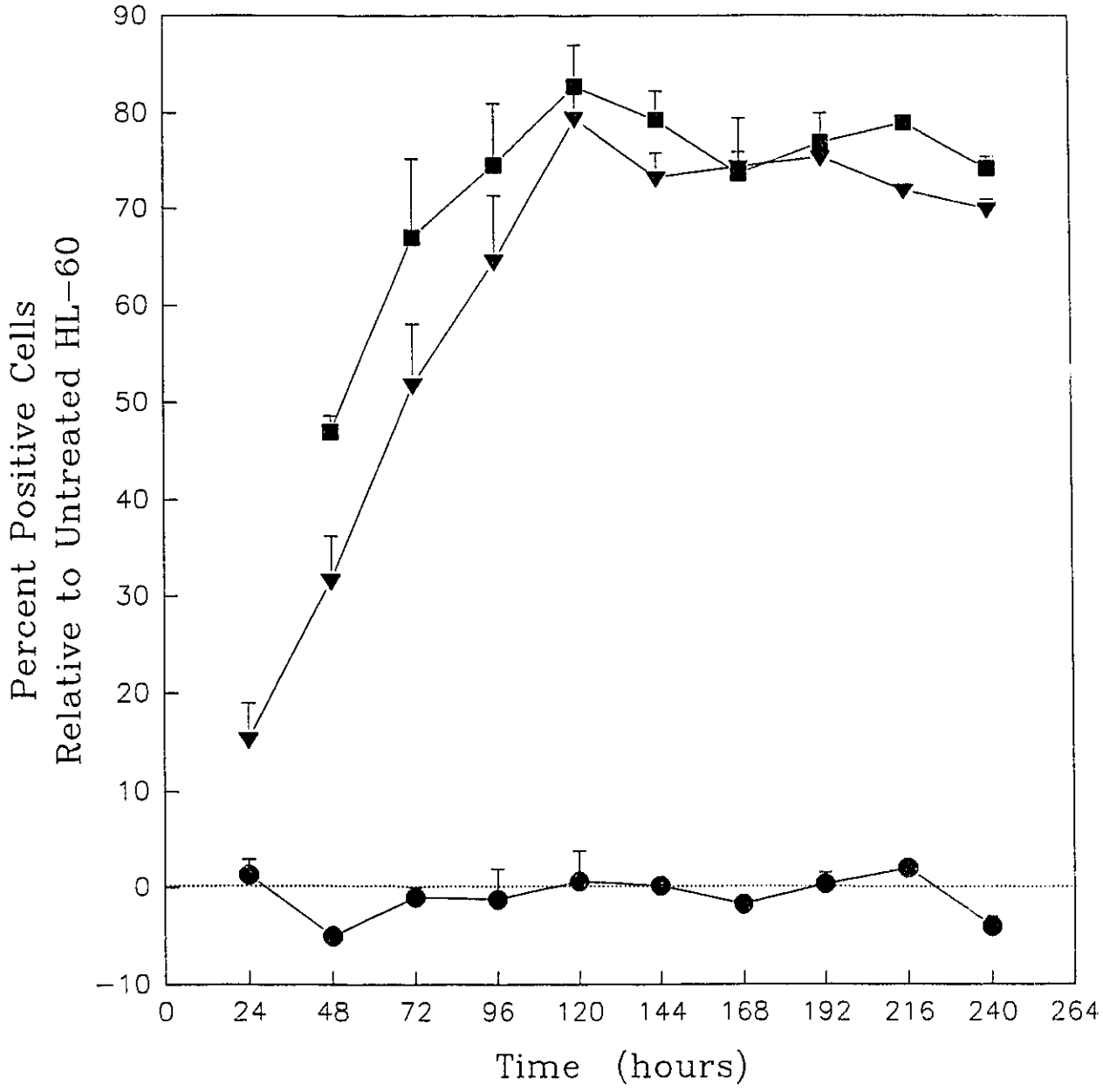
Speckled staining at the plasma membrane is evident in most of the cells pictured in figures 7F and 7H. 63D3 staining was negative in all TPA-treated cells. An example from the adherent subpopulation is given in panels 7I and 7J. Macrophages also did not express the 63D3 antigen, as depicted in panels 7K and 7L.

The kinetics of monocyte marker 63D3 expression during induction of differentiation by 5×10^{-7} M vitamin D_3 was followed by cell counts and the results of this analysis are presented in figure 8. The level of expression of the 63D3 antigen in the ethanol-treated control population never varied significantly from that seen in untreated HL-60 cells. In contrast, the proportion of 63D3-positive cells in the treated cell subpopulations rose dramatically throughout the first 120 hours of vitamin treatment. This increase occurred at a similar rate in both nonadherent and adherent cells, as seen by the nearly parallel slopes of the plots on the graph. After 120 hours, expression of 63D3 antigen plateaued at 75 - 80% above control values. This high level of expression continued throughout the duration of the induction period. Throughout the entire differentiation period, the two treated subpopulations did not differ significantly from one another except at the 48 hour time point, as seen by the error bars on the individual data points and confirmed by t-tests comparing each of the time points separately. The discrepancy at 48 hours was due to a more rapid initiation of marker expression in the adherent cells. By the next time point, at 72 hours, the difference between the treated subpopulations was no longer significant. Treating HL-60 cells with vitamin D_3 gave rise to expression of monocyte-specific protein within 24 hours. By 120 hours of treatment almost all cells in the exposed population expressed the marker detected by the 63D3 antibody, regardless of their state of attachment to the substratum. This high level of expression continued throughout the remainder of the

Figure 8. Kinetic analysis of monocyte marker 63D3 expression in HL-60 cells during induction of differentiation by 5 x 10^{-7} M vitamin D_3. Cells expressing 63D3 as detected by immunofluorescent staining were quantified as a percentage of the total cell population. Results are expressed as the percentage of positive cells in treated cultures relative to those in untreated HL-60 control cultures. The dashed line indicates the zero point established by the level of protein expression in these control cultures. Results are the average of triplicate experiments. Error bars indicate standard error of the multiple measurements. Filled circles indicate results for ethanol-treated controls, filled triangles those of vitamin-treated cells which remained nonadherent and filled squares the results for vitamin-treated cells which became adherent.


Percent Positive Cells
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72
96
120
144
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Time (hours)

differentiation period.

The kinetics of 63D3 expression in HL-60 cells induced to differentiate with 3 x 10^{-8}M TPA was also assessed by cell counts. Figure 9 presents the results of this analysis. Neither the ethanol treated controls nor the TPA-treated cell populations expressed the 63D3 marker at a rate significantly different from untreated HL-60 cells. This was confirmed by t-tests of the individual data points. Treatment with TPA did not induce expression of the 63D3 antigen in HL-60 cells.

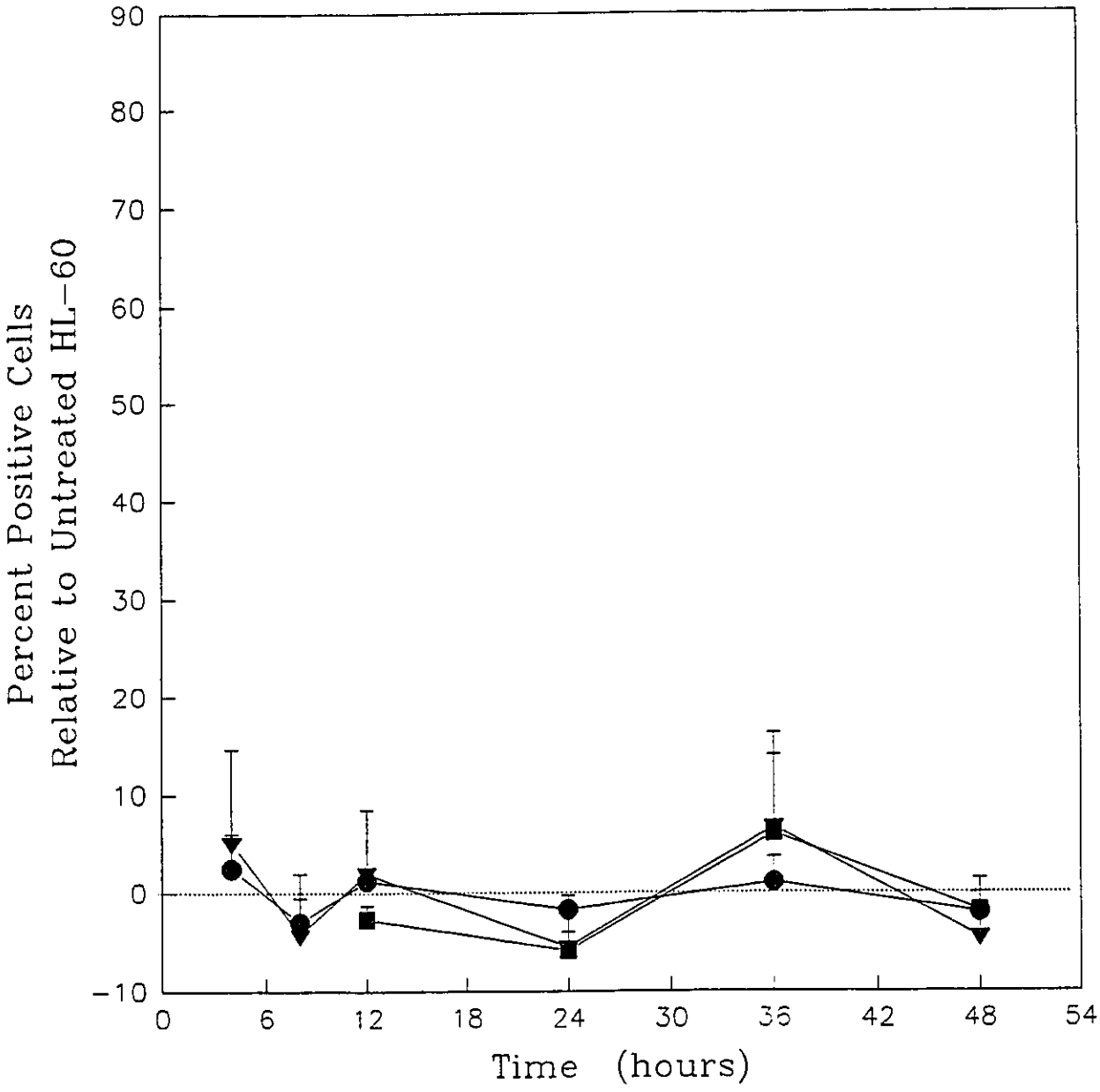
The expression of 63D3 protein in mouse peritoneal macrophages was also examined to provide a normal cell reference for interpretation of the HL-60 cell findings. As can be seen in table 1, only a small minority of macrophages representative of both the inactivated state (24 hours in culture) or of the activated state (168 hours in culture) expressed this monocyte-specific marker. Clearly 63D3 expression is not typical of macrophages.

B) Microscopic Detection and Kinetic Analysis of TR Expression

The expression of TR protein in differentiating HL-60 cells was detected using immunofluorescent staining with the monoclonal antibody L5.1 as described in Materials and Methods. The kinetics of TR expression during treatment with either 5 x 10^{-7}M vitamin D_3 or 3 x 10^{-8}M TPA was assessed by cell counts. This approach was also used to monitor TR expression in mouse peritoneal macrophages.

Examples of TR staining patterns seen in control and induced HL-60 cell populations are presented in figure 10. Panels 10A and 10B show untreated HL-60 cells, while 10C and 10D depict ethanol-treated controls. TR-positive cells were common in these populations. They

Figure 9. Kinetic analysis of monocyte marker 63D3 expression in HL-60 cells during induction of differentiation by 3 x 10^{-8} M TPA. Cells expressing 63D3 as detected by immunofluorescent staining were quantified as a percentage of the total cell population. Results are expressed as the percentage of positive cells in treated cultures relative to those in untreated HL-60 control cultures. The dashed line indicates the zero point established by the level of protein expression in these control cultures. Results are the average of triplicate experiments. Error bars indicate standard error of the multiple measurements. Filled circles indicate results for ethanol-treated controls, filled triangles those of TPA-treated cells which remained nonadherent and filled squares the results for TPA-treated cells which became adherent.


Percent Positive Cells
Relative to Untreated HL-60
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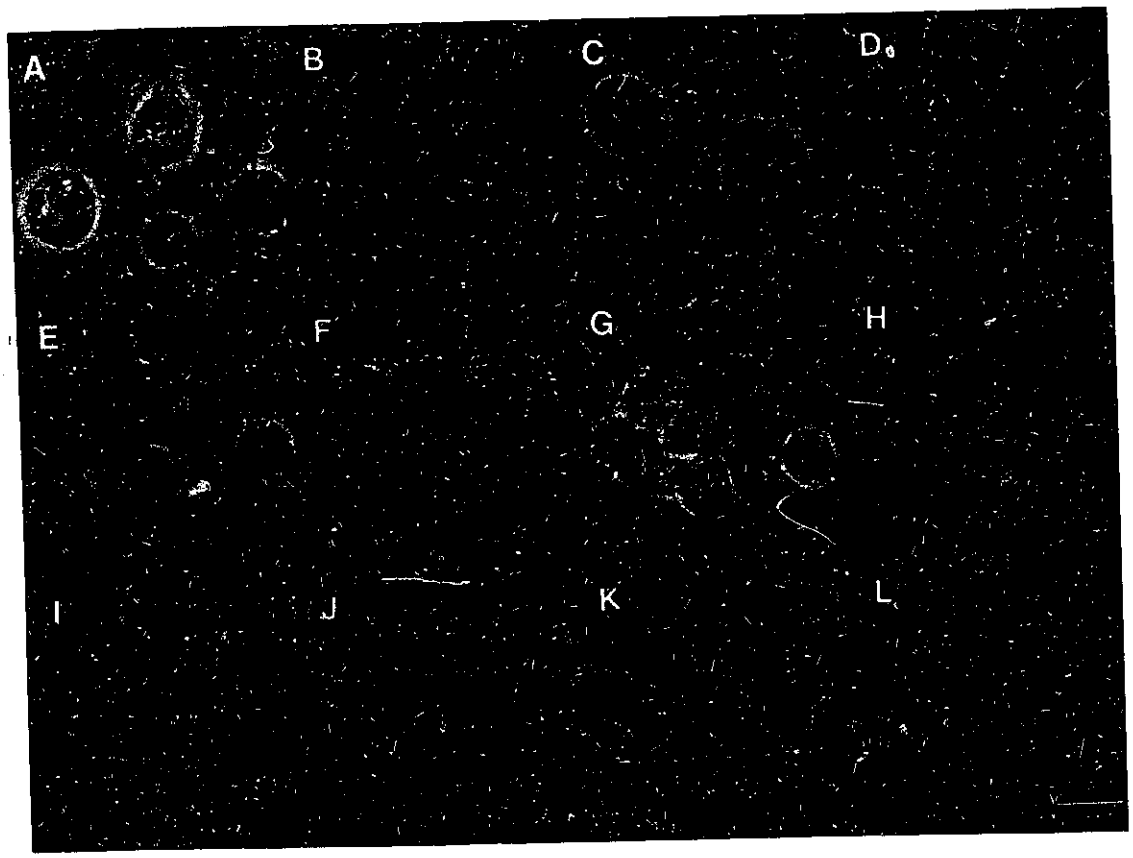
Time (hours)

Figure 10. Detection of transferrin receptor protein in HL-60 cells and mouse peritoneal macrophages. Transferrin receptor was detected using immunofluorescent staining with the mouse monoclonal antibody L5.1, visualised with an FITC-conjugated goat anti-mouse IgG antibody. Typical results for untreated HL-60 (A and B), control HL-60 treated with ethanol alone for 24 hours (C and D), HL-60 treated with vitamin D_3 for 240 hours (E,F,G and H), HL-60 treated with TPA for 24 hours (I and J) and peritoneal macrophages kept in primary culture for 168 hours (K and L) are shown. Results for those HL-60 which remained in suspension after vitamin D_3 treatment (E and F) and those which became adherent (G,H,I and J) are both included. Phase contrast (A,C,E,G,I and K) and corresponding fluorescent images (B,D,F,H,J and L) are shown.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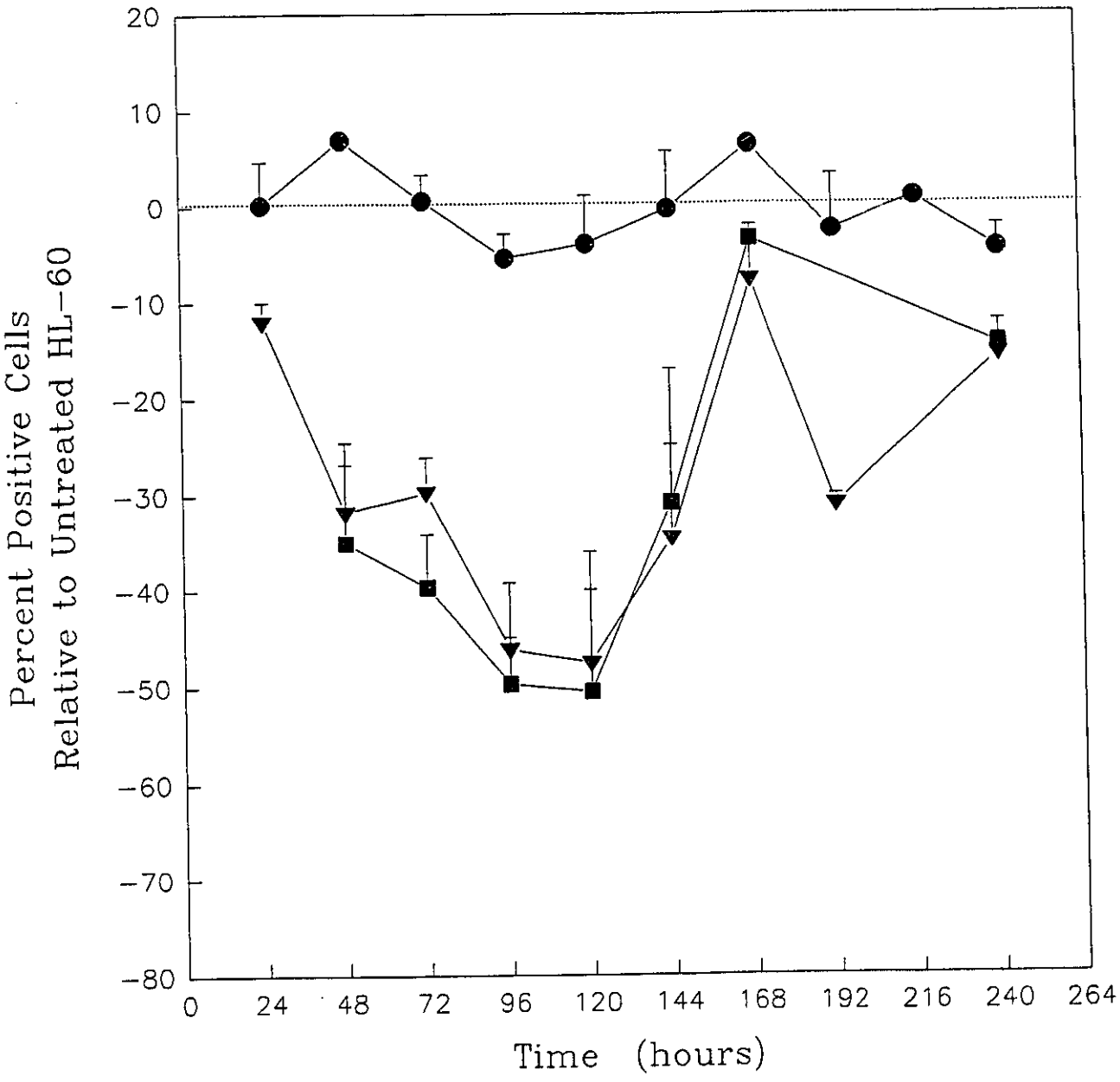
tended to stain very brightly over an area of the cell surface. This area varied in size from a small "cap", as seen in figure 10D, to almost the entire cell circumference, as depicted in figure 10B. In HL-60 cells induced to differentiate with vitamin D_3 the expression of transferrin receptor dropped (figures 10E-10H). Both the proportion of cells expressing the protein and the amount of staining seen on positive cells decreased. Receptor expression became limited to several small, discrete patches at the cell surface, as seen in the left hand cell in figure 10F. It was difficult to reproduce this staining pattern photographically, as the patches rarely lay in the same plane of focus on these round cells. Despite the sparse staining pattern, results could be interpreted with confidence because of the extremely low level of nonspecific background present. Although the expression of receptor became very limited on any individual cell, it was still clearly different from those neighbours which were negative. Panels 10I and 10J depict TPA-treated HL-60 cells; they clearly did not express transferrin receptor. Isolated mouse peritoneal macrophages are depicted in figures 10K and 10L. These cells expressed transferrin receptor in a pattern similar to that seen in the vitamin D_3-treated HL-60; small, discrete patches of receptors were seen on the cell surface (figure 10L). Photographic representation was more accurate for this cell type than for the vitamin-treated cells because the macrophages were flat, adherent cells whose surface lay largely within one plane of focus.

The kinetics of TR expression in HL-60 cells induced to differentiate by 5 x 10^{-7}M vitamin D_3 was assessed by cell counts and the results expressed in figure 11. 55-70% of the untreated HL-60 cell population expressed the TR protein. The exact level varied with the quality of culture conditions, as described for lamins A and C. The baseline level of expression established by untreated cells is indicated by the dashed line. Clearly, ethanol-treated control

Figure 11. Kinetic analysis of transferrin receptor expression in HL-60 cells during induction of differentiation by 5 x 10^{-7} M vitamin D_3. Cells expressing transferrin receptor as detected by immunofluorescent staining were quantified as a percentage of the total cell population. Results are expressed as the percentage of positive cells in treated cultures relative to those in untreated HL-60 control cultures. The dashed line indicates the zero point established by the level of protein expression in these control cultures. Results are the average of triplicate experiments. Error bars indicate standard error of the multiple measurements. Filled circles indicate results for ethanol-treated controls, filled triangles those of vitamin-treated cells which remained nonadherent and filled squares the results for vitamin-treated cells which became adherent.

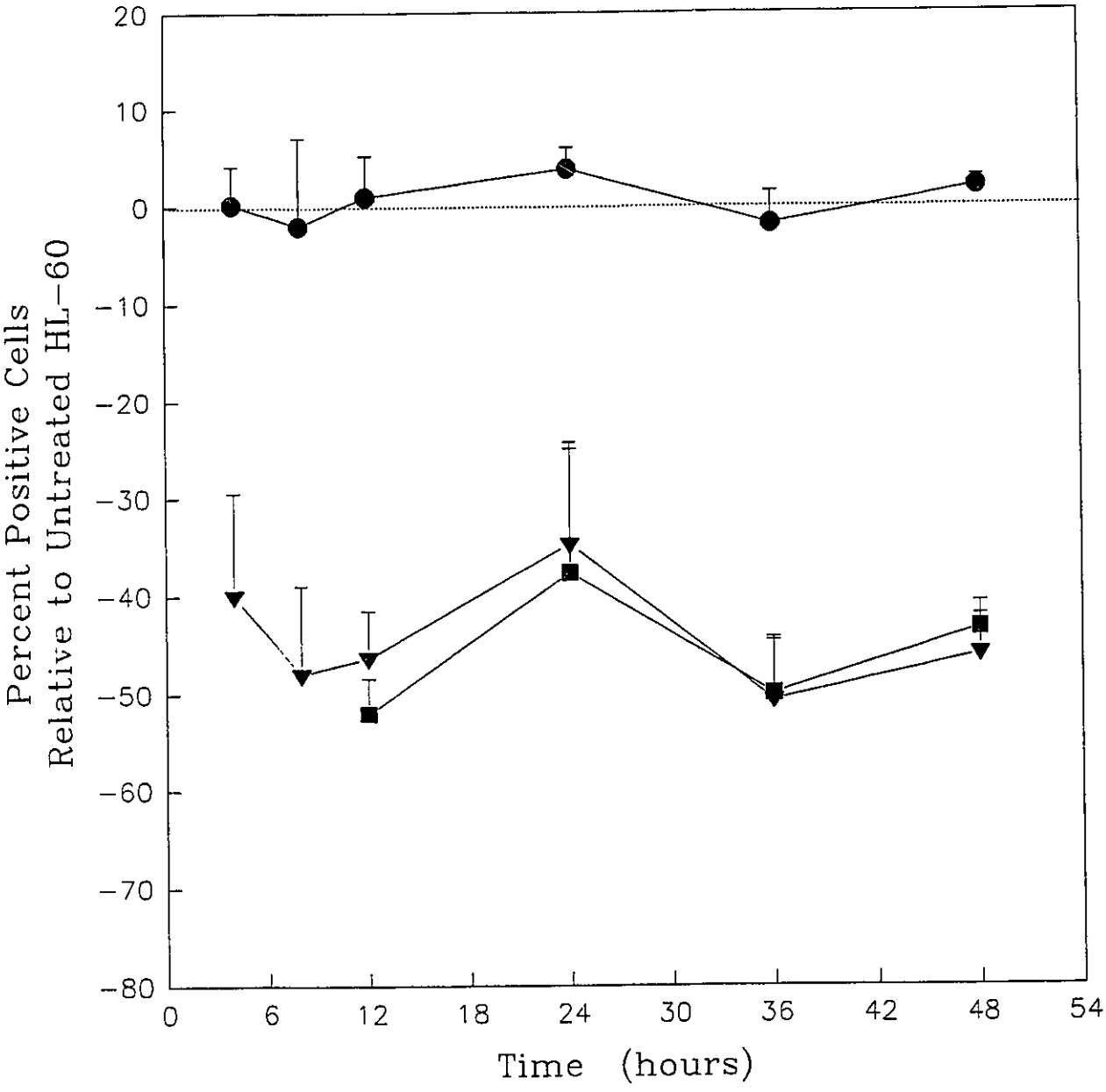

Percent Positive Cells
Relative to Untreated HL-60
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
0
24
48
72
96
120
144
168
192
216
240
264
Time (hours)

cells did not differ significantly from their untreated counterparts during the course of induction. In contrast, expression of TR in both vitamin-treated subpopulations fell throughout the first half of the induction period, up to the 120 hour time point. From 120 hours to 168 hours the trend was reversed; both the suspension and adherent populations had increases in the proportion of cells expressing TR. From the 168 hour time point to the 240 hour mark, the level of TR expression in the treated cells remained 5 - 15% below that of the control populations. Throughout these changes, no significant difference was noted between the suspension and adherent subpopulations. Induction of HL-60 with vitamin D_3 led to a transient decrease in TR expression during the first half of the treatment period, followed by an increase and plateau at near-control levels.

The results of kinetic analysis of TR expression in TPA-treated HL-60 cells are depicted in figure 12. The expression of TR in the ethanol-treated control population did not differ from that seen in the untreated HL-60 cell population. Both TPA-treated subpopulations had a dramatic drop in TR expression to 40 - 50% below control level. This drop occurred immediately upon induction with TPA; it was evident by the 4 hour time point. Thereafter the level of TR expression did not change markedly. Results for the suspension and adherent subpopulations did not differ significantly from each other. Induction of HL-60 cells with 3 x 10^{-8}M TPA led to an immediate drop in expression of TR receptor.

In order to provide a normal physiological control for purposes of comparison to the treated HL-60 cell populations, mouse peritoneal macrophage primary cultures were stained for TR. As seen in table 1, the vast majority of both newly isolated macrophages (24 hours in culture) and activated macrophages (168 hours in culture) expressed the TR protein. This result

Figure 12. Kinetic analysis of transferrin receptor expression in HL-60 cells during induction of differentiation by 3 x 10^{-8} M TPA. Cells expressing transferrin receptor as detected by immunofluorescent staining were quantified as a percentage of the total cell population. Results are expressed as the percentage of positive cells in treated cultures relative to those in untreated HL-60 control cultures. The dashed line indicates the zero point established by the level of protein expression in these control cultures. Results are the average of triplicate experiments. Error bars indicate standard error of the multiple measurements. Filled circles indicate results for ethanol-treated controls, filled triangles those of TPA-treated cells which remained nonadherent and filled squares the results for TPA-treated cells which became adherent.


Percent Positive Cells
Relative to Untreated HL-60
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
0
6
12
18
24
30
36
42
48
54
Time (hours)

confirmed literature reports that TR expression is characteristic of the macrophage cell type (Röber et al., 1990b).

POPULATION GROWTH, DNA REPLICATION AND CELL CYCLE DISTRIBUTION DURING DIFFERENTIATION OF HL-60 CELLS

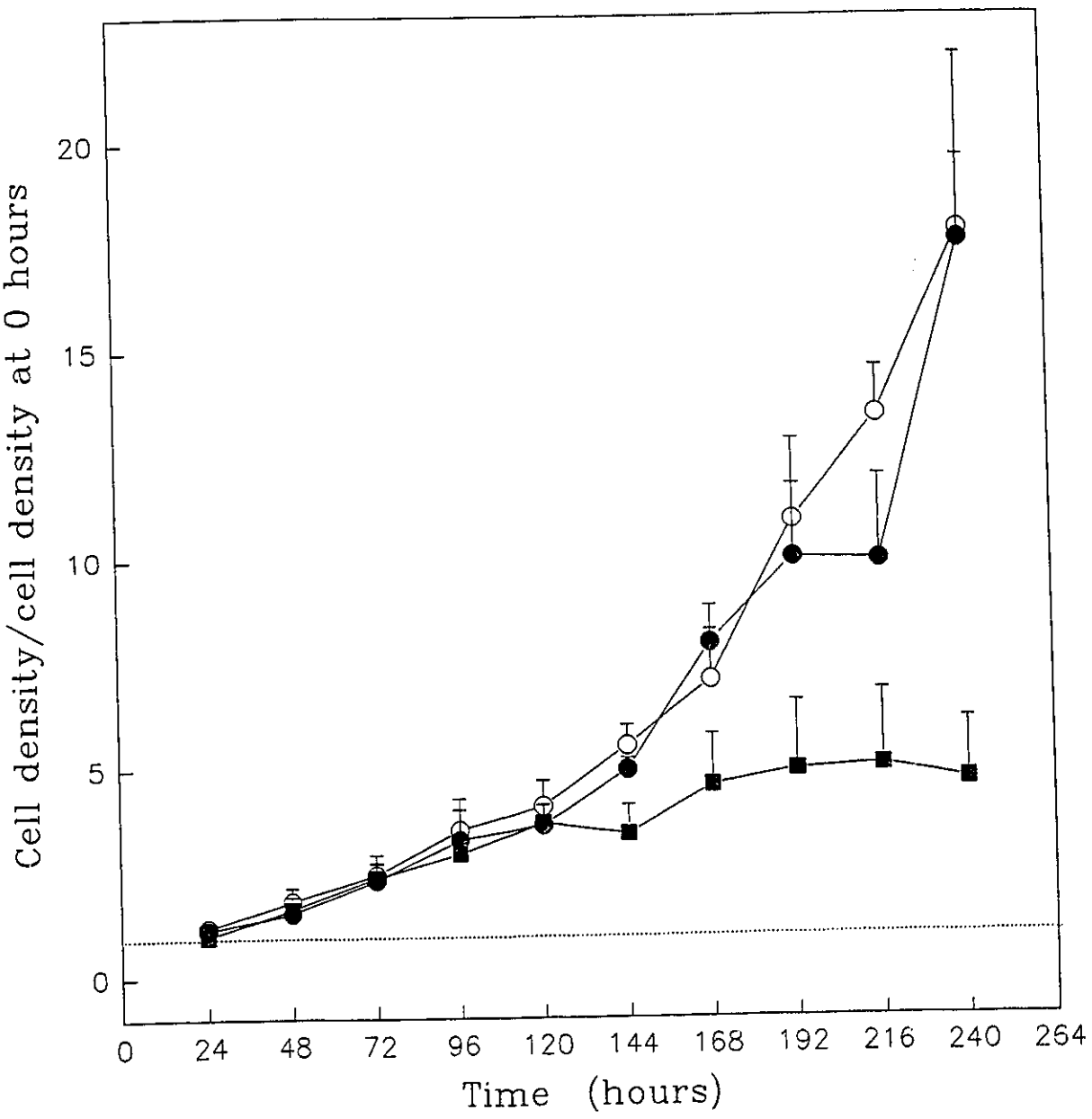
One of the hallmarks of differentiation in HL-60 cells is exit from the cell cycle and entry into G_0/G_1. It was therefore expected that the response of HL-60 cells to induction of differentiation would include cessation of cell growth. Population growth, incorporation of ^{3}H-thymidine into replicating DNA and cell cycle distribution were all monitored throughout differentiation in order to assess the effect(s) of vitamin D_3 and TPA on the replicative status of the cells.

A) Analysis of Population Growth

Growth of the HL-60 cell population was monitored throughout the induction of differentiation by haemocytometer counts of trypan-blue excluding cells, as described in Materials and Methods.

The effects of vitamin D_3 treatment on growth of the HL-60 cell population are documented in figure 13. Results are reported as the cell density at a given time point relative to the initial ceil density. The dashed line indicates a value of 1, or cell density equal to the starting concentration. Both the treated and control populations showed no significant change

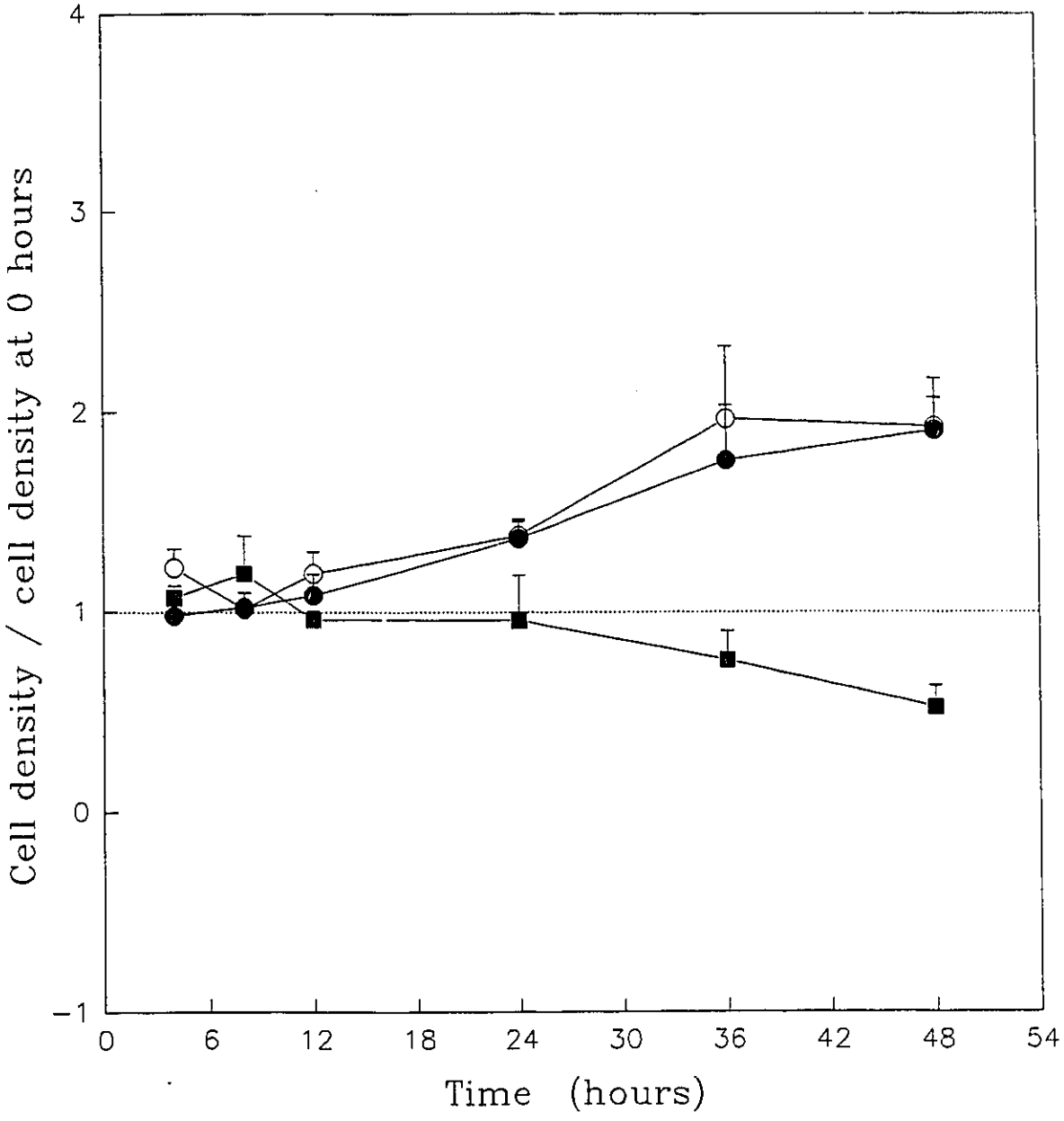
Figure 13. Growth of the HL-60 cell population during induction of differentiation by 5 x 10^{-7} M vitamin D_3. Population growth was monitored via haemocytometer counts of trypan blue-excluding cells. Results are expressed as the cell number at a given time point relative to the initial cell number. The dashed line indicates a value of 1, or no growth beyond the initial cell density. Results are the average of triplicate experiments. Error bars represent the standard error of the multiple measurements. Open circles indicate results for untreated HL-60 cells, filled circles those for ethanol-treated controls, and filled squares the results for vitamin-treated HL-60.


Cell density/cell density at 0 hours
20
15
10
5
0
0
24
48
72
96
120
144
168
192
216
240
264
Time (hours)

in cell density during the first 24 hours of culture. HL-60 cells routinely showed a lag in growth after any manipulation of culture conditions. From 24 to 120 hours, all cell populations doubled every 48 hours. After this time point, a divergence of the growth curve of treated cells from that of control populations can be seen. Both the untreated HL-60 and the ethanol-treated control cells continued to double every 48 hours, never differing significantly from each other. A slight decrease in their growth rate occurred in the last two days of the induction period; by this time the cultures were quite crowded. In contrast the vitamin-treated cell population, which included both the suspension and adherent subpopulations, showed no significant change in cell density after the 120 hour time point. Growth of cells exposed to 5 x 10^{-7}M vitamin D_3 was arrested during the latter half of the induction period.

The growth curve of HL-60 cells in the presence of 3 x 10^{-8}M TPA is presented in figure 14. For the first 12 hours there was no significant change in cell density in any of the populations. By 24 hours, the untreated HL-60 and ethanol-treated control cells showed only a marginal increase in cell density, far less than a doubling of cell number. From 24 to 48 hours, the density of both the untreated and ethanol-treated control populations almost doubled. Throughout the treatment period these two populations did not differ significantly from each other. The cell density of the TPA-treated cell population, which included both the suspension and adherent subpopulations, showed no significant change up to the 24 hour time point. However, it diverged significantly from control values during the latter half of the induction period. Treated cell density decreased steadily from 24 to 48 hours, where it was approximately 60% of the initial cell density. Cells treated with 3 x 10^{-8}M TPA not only showed inhibition of any population growth, they had a marked decrease in viability after 24 hours of treatment.

Figure 14. Growth of the HL-60 cell population during induction of differentiation by 3 x 10^{-8} M TPA. Population growth was monitored via haemocytometer counts of trypan blue-excluding cells. Results are expressed as the cell number at a given time point relative to the initial cell number. The dashed line indicates a value of 1, or no growth beyond the initial cell density. Results are the average of triplicate experiments. Error bars represent the standard error of the multiple measurements. Open circles indicate results for untreated HL-60 cells, filled circles those of ethanol-treated controls and filled squares the results for TPA-treated cells.

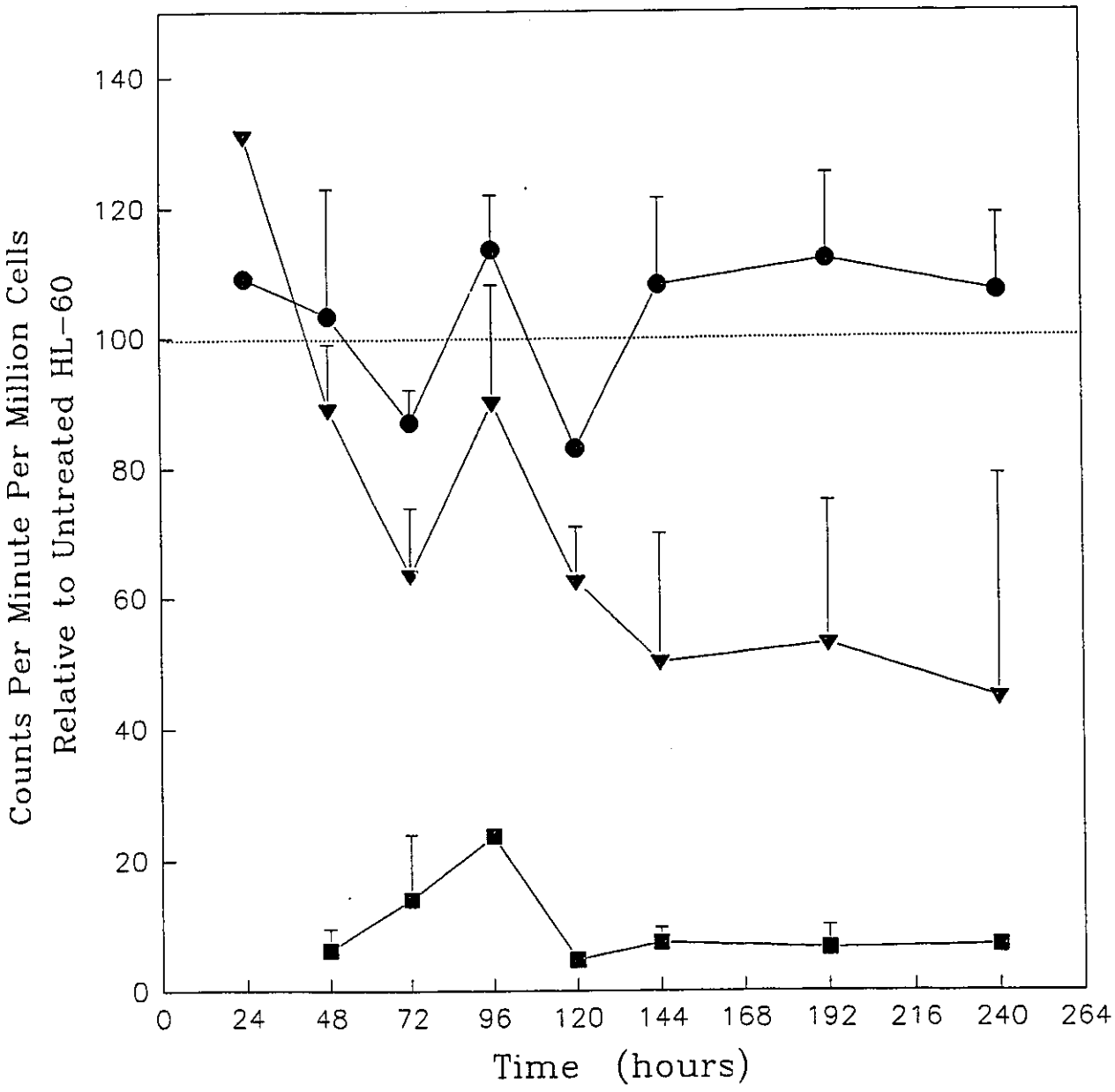

Cell density / cell density at 0 hours
4
3
2
1
0
−1
0
6
12
18
24
30
36
42
48
54
Time (hours)

B) Detection of DNA Replication

DNA replication in HL-60 cells was monitored throughout the treatment period by pulse-labelling with ^{3}H-thymidine, as described in Materials and Methods. The amount of radioactive material present in harvested cellular DNA was then assessed with a scintillation counter.

The effects of vitamin D_3 treatment on incorporation of ^{3}H-thymidine into DNA in HL-60 cells are presented in figure 15. Results are expressed as the amount of radioactivity present relative to the level detected in untreated HL-60 cells at the same time point. The dashed line indicates the 100% level established by these control values. The ethanol-treated control population did not differ significantly from this baseline throughout the induction period. The nonadherent treated subpopulation cycled from above to below control levels during the first 96 hours of the treatment period. These changes parallelled smaller, nonsignificant trends seen in the ethanol-treated control population during this same period. This pattern may reflect partial synchrony of the cells due to culture manipulations performed during experimental setup. From 120 hours to the final 240 hour time point, the nonadherent treated cells showed a partial inhibition of thymidine incorporation, to approximately 50% of the level seen in controls. Although there was considerable interexperimental variation in the exact levels of ^{3}H-thymidine uptake, the trend was significant, as demonstrated by the error bars on the individual data points. The vitamin-induced cells which became adherent had a distinct pattern of thymidine incorporation; they were extremely inhibited relative to the control cultures. Up to the 96 hour time point they incorporated less than 22% of control levels of thymidine, the exact values undulating in a pattern similar to the other populations studied. From 120 to 240 hours they took up less than 5% of control levels of ^{3}H-thymidine . This trend was very consistent, as

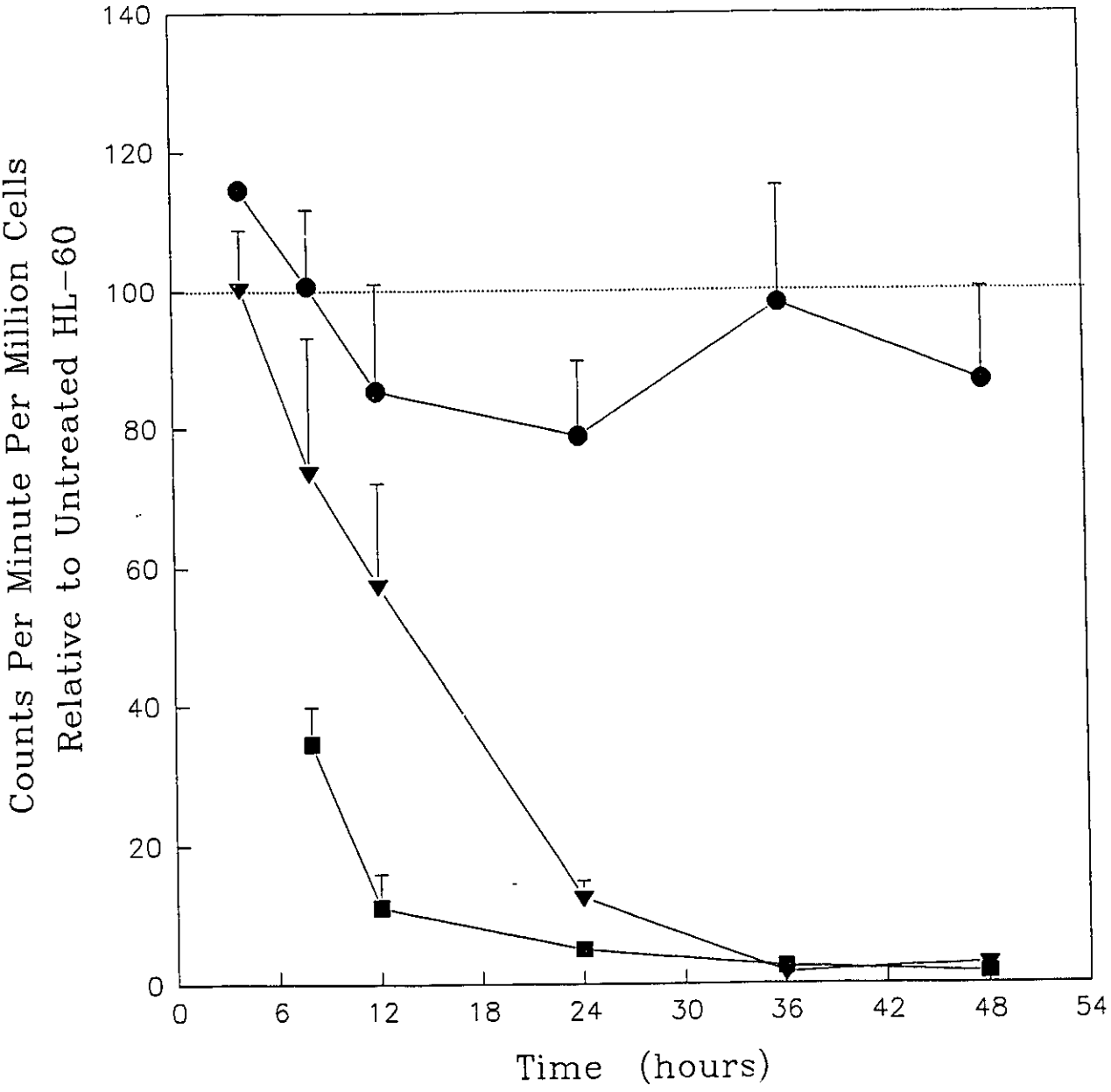
Figure 15. DNA replication in HL-60 cells during induction of differentiation by 5 x 10^{-7} M vitamin D_3. The extent of DNA replication was monitored by assessing the incorporation of ^{3}H-thymidine into cellular DNA during a ninety minute pulse. Results are expressed as the extent of labelled thymidine incorporation relative to untreated HL-60 control cells. The dashed line indicates the 100% mark established by the incorporation level of these control cells. Results are the mean of triplicate experiments. Error bars represent the standard error of the multiple measurements. Filled circles indicate results for ethanol-treated controls, filled triangles those of vitamin-treated cells which remained nonadherent and filled squares the results for vitamin-treated cells which became adherent.


Counts Per Minute Per Million Cells
Relative to Untreated HL-60
140
120
100
80
60
40
20
0
0
24
48
72
96
120
144
168
192
216
240
264
Time (hours)

evidenced by the small error bars on the individual data points. In the presence of 5 x 10^{-7}M vitamin D_3, incorporation of ^{3}H-thymidine into replicating DNA in HL-60 cells was partially inhibited at later time points in the nonadherent subpopulation and fully inhibited throughout the differentiation period in the adherent subpopulation.

Figure 16 depicts ^{3}H-thymidine incorporation into the DNA of HL-60 cells induced to differentiate by 3 x 10^{-8}M TPA. The ethanol-treated control cells did not differ significantly from the untreated HL-60 throughout the induction period, although the error bars on individual data points testify to the marked interexperimental variation in incorporation seen. TPA-treated cells which remained in suspension began to show inhibition of thymidine incorporation after 4 hours. By the 12 hour time point presence of the label was significantly reduced to approximately 60% of control level. The error bars on these early data points demonstrate a degree of interexperimental variability in the exact amount of inhibition, despite the overall significance of the trend. From 24 to 48 hours of treatment, the nonadherent subpopulation incorporated virtually no ^{3}H-thymidine into its DNA; values were very consistently less than 10% of controls. The adherent TPA-treated cells displayed more marked effects on DNA replication. This subpopulation was inhibited in its incorporation of ^{3}H-thymidine into DNA by 12 hours of treatment, to 35% of control levels. From 12 hours through to the end of the induction period, thymidine incorporation was reproducibly less than 10% of control levels. TPA-induced differentiation of HL-60 cells resulted in complete inhibition of ^{3}H-thymidine incorporation into replicating DNA in all cells, although the nonadherent subpopulation demonstrated this effect fully only after a 24 hour lag.

Figure 16. DNA replication in HL-60 cells during induction of differentiation by 3 x 10^{-8} M TPA. The extent of DNA replication was monitored by assessing the incorporation of ^{3}H-thymidine into cellular DNA during a ninety minute pulse. Results are expressed as the extent of labelled thymidine incorporation relative to untreated HL-60 control cells. The dashed line indicates the 100% mark established by the incorporation level of these control cells. Results are the mean of triplicate experiments. Error bars represent the standard error of the multiple measurements. Filled circles indicate results for ethanol-treated controls, filled triangles those of TPA-treated cells which remained nonadherent and filled squares the results for TPA-treated cells which became adherent.

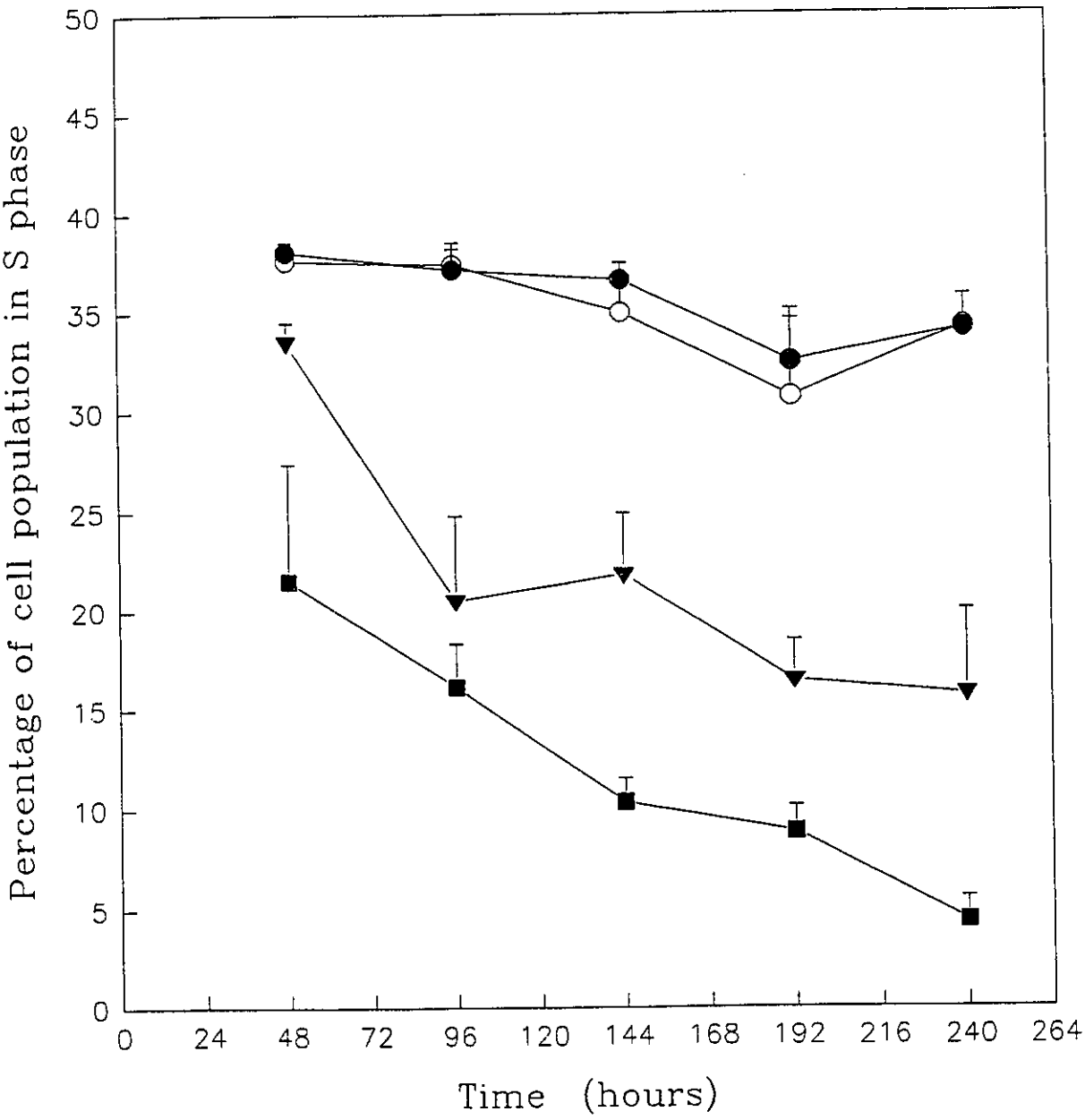

Counts Per Minute Per Million Cells
Relative to Untreated HL-60
140
120
100
80
60
40
20
0
0
6
12
18
24
30
36
42
48
54
Time (hours)

C) Analysis of Cell Cycle Distribution by Flow Cytometry

The effects of vitamin D_3 or TPA induction on HL-60 cell cycle distribution were examined using flow cytometric analysis of propidium iodide-stained cells, as described in Materials and Methods. Fluorescence of each cell, indicative of the amount of DNA present, was quantified using a Coulter Profile II flow cytometer. Histograms of the population distribution of DNA content were examined using Multicycle, a commercial software package for cell cycle analysis, to determine the proportion of cells in G_1, S and G_2/M.

The effects of HL-60 cell induction by 5 x 10^{-7}M vitamin D_3 on the percentage of cells in the population in S phase are depicted in figure 17. Both untreated HL-60 cells and ethanol-treated control populations showed no significant change in the distribution of cells in S phase throughout the differentiation period; between 32% and 38% of control cells were found to be in S phase. The consistency of these values is indicated by the small size of the error bars in the data points. The vitamin D_3-treated cells evidenced a marked shift in the percentage of cells in S phase throughout the course of differentiation. In the nonadherent subpopulation, the percentage of cells in S phase dropped significantly from 34% at 48 hours to 22% at 96 hours. From 96 to 240 hours there was an apparent slight decrease to approximately 16% of cells in S phase, but the error bars on these data points indicate that this small change was not significant. The adherent subpopulation of treated cells had a distinct S phase distribution. They displayed a significant drop to 22% percent of cells in S phase by 48 hours, followed by a steady decline until 240 hours, when only 5% of the population remained in S phase. Vitamin D_3 treatment led to a reduction in the proportion of nonadherent cells in S phase by almost half after 96 hours of treatment. In the adherent subpopulation there was continual decrease in the

Figure 17. Distribution of HL-60 cells in S phase during induction of differentiation by 5×10^{-7} M vitamin D_3. The DNA content of individuals in treated and control populations was assessed by flow cytometry of propidium iodide-stained cells and the percentage of cells in S phase determined using Multicycle, a standard cell cycle analysis software package. Results plotted are the mean of triplicate experiments. Error bars represent the standard error of the multiple measurements. Open circles indicate results for untreated HL-60 cells, filled circles designate results for ethanol-treated controls, filled triangles those of vitamin-treated cells which remained nonadherent and filled squares the results for vitamin-treated cells which became adherent.

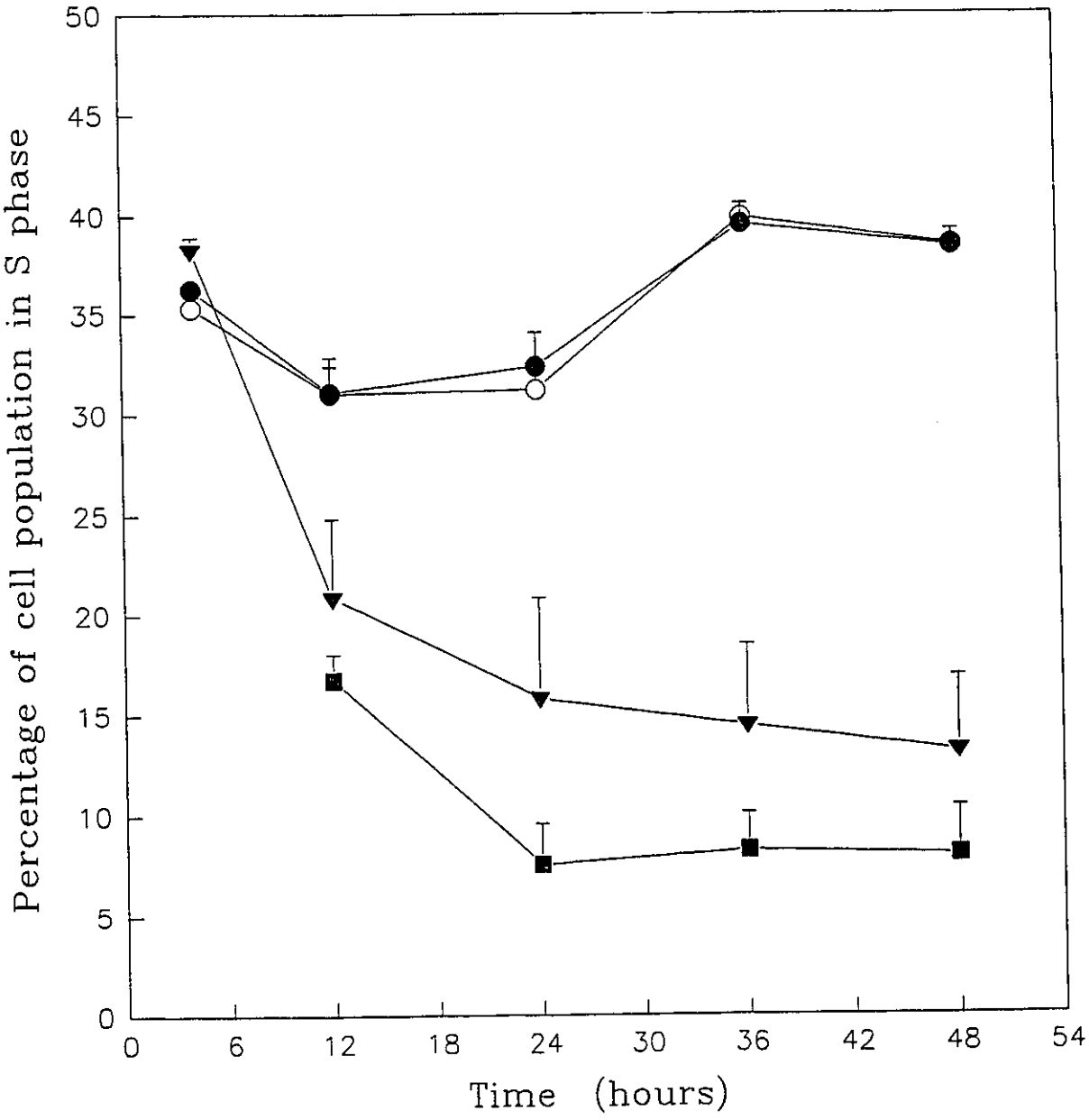

Percentage of cell population in S phase
50
45
40
35
30
25
20
15
10
5
0
0
24
48
72
96
120
144
168
192
216
240
264
Time (hours)

fraction of cells in S, evident by 48 hours of induction.

Figure 18 depicts the effects of TPA treatment of HL-60 on the proportion of cells in S phase. Once again, the untreated HL-60 and ethanol-treated controls did not differ significantly from each other; 31% - 35% of these cells were in S phase during the first 24 hours of treatment, with a slight but significant rise to 38 -40% of cells in S by 48 hours. Interexperimental variation in these values was minimal. Induction with TPA gave rise to dramatic shifts in this distribution. The nonadherent subpopulation was comparable to controls at 4 hours, but then dropped to 22% of cells in S by 12 hours of treatment. From 12 to 24 hours, a slower decline to 15% of cells in S phase occurred. No further significant changes in this distribution were seen. In the adherent subpopulation only 16% of the cells were in S phase by the 12 hour time point. This proportion decreased further to 8% in S by 24 hours, after which no significant changes in cell cycle distribution were seen. Although the proportion of cells in S phase was apparently lower in the adherent than in the nonadherent subpopulation after 12 hours, an examination of the error bars on the individual data points shows that this difference was not statistically significant. Induction of HL-60 cells with 3 x 10^{-8}M TPA led to a dramatic decrease of the proportion of cells in S phase. This decrease was evident by 12 hours of treatment and reached a peak by the 24 hour time point, when the proportion of cells in S phase was reduced to approximately 10%.

The reduced proportion of cells in S phase in populations of treated HL-60 cells implied that cell cycle block(s) had occurred. In order to detect and compare the blockages in cell cycle progression due to vitamin D_3 and TPA, the distribution of cells in G_1 and G_2/M phases was monitored throughout treatment.

Figure 18. Distribution of HL-60 cells in S phase during induction of differentiation by 3 x 10^{-8} M TPA. The DNA content of individuals in treated and control populations was assessed by flow cytometry of propidium iodide-stained cells and the percentage of cells in S phase determined using Multicycle, a standard cell cycle analysis software package. Results plotted are the mean of triplicate experiments. Error bars represent the standard error of the multiple measurements. Open circles indicate results for untreated HL-60 cells, filled circles designate results for ethanol-treated controls, filled triangles those of TPA-treated cells which remained nonadherent and filled squares the results for TPA-treated cells which became adherent.


Percentage of cell population in S phase
50
45
40
35
30
25
20
15
10
5
0
0
6
12
18
24
30
36
42
48
54
Time (hours)

The effects of 5 x 10^{-7}M vitamin D_3 on the distribution of HL-60 cells in G_1 are presented in figure 19. Untreated HL-60 cells and ethanol-treated control populations contained 47 - 49% G_1 cells throughout the induction period. The overlap of these two plots and the minimal error bars on the individual data points testify to the consistency of these results. The vitamin-treated, nonadherent subpopulation contained a similar fraction of G_1 cells up to 48 hours of treatment. From 48 to 96 hours, a rapid rise to 65% G_1 cells occurred. Thereafter, a slight rise to 70% of cells in G_1 by 240 hours was seen. The error bars on these data points indicate that the latter small change was not significant. The treated cells which became adherent had a distinct response. At 48 hours, 67% of this subpopulation was in G_1. This fraction increased steadily throughout the induction period to reach 83% of cells in G_1 by 240 hours. Following vitamin D_3 treatment, nonadherent HL-60 cells demonstrated an increase in the fraction of the population in G_1 between 48 and 96 hours, followed by a plateau. In contrast, the adherent subpopulation had an accumulation of cells in G1 from the earliest time point which increased throughout the induction period.

The effects of vitamin D_3 treatment on the proportion of HL-60 cells in G_2/M was also noted, and the results depicted in figure 20. 13-15% of untreated and ethanol-treated HL-60 cells were consistently found in the G_2 fraction throughout the induction period. The proportion of G_2 cells in both vitamin-induced subpopulations was very close to these control values. The nonadherent subpopulation varied from 17% G_2 cells at 48 hours to 13% of cells in G_2 by 240 hours. The fraction of treated, adherent cells in G_2 rose from 10% at 48 hours to 13% at 240 hours. As the error bars on the data points indicate, these slight differences between the control and treated populations were, for the most part, not significant. Moreover, no trend is evident

Figure 19. Distribution of HL-60 cells in the G_1 phase of the cell cycle during induction of differentiation by 5 x 10^{-7} M vitamin D_3. The DNA content of individuals in treated and control populations was assessed by flow cytometry of propidium iodide-stained cells and the percentage of cells in G_1 phase determined using Multicycle, a standard cell cycle analysis software package. Results plotted are the mean of triplicate experiments. Error bars represent the standard error of the multiple measurements. Open circles indicate results for untreated HL-60 cells, filled circles designate results for ethanol-treated controls, filled triangles those of vitamin-treated cells which remained nonadherent and filled squares the results for vitamin-treated cells which became adherent.

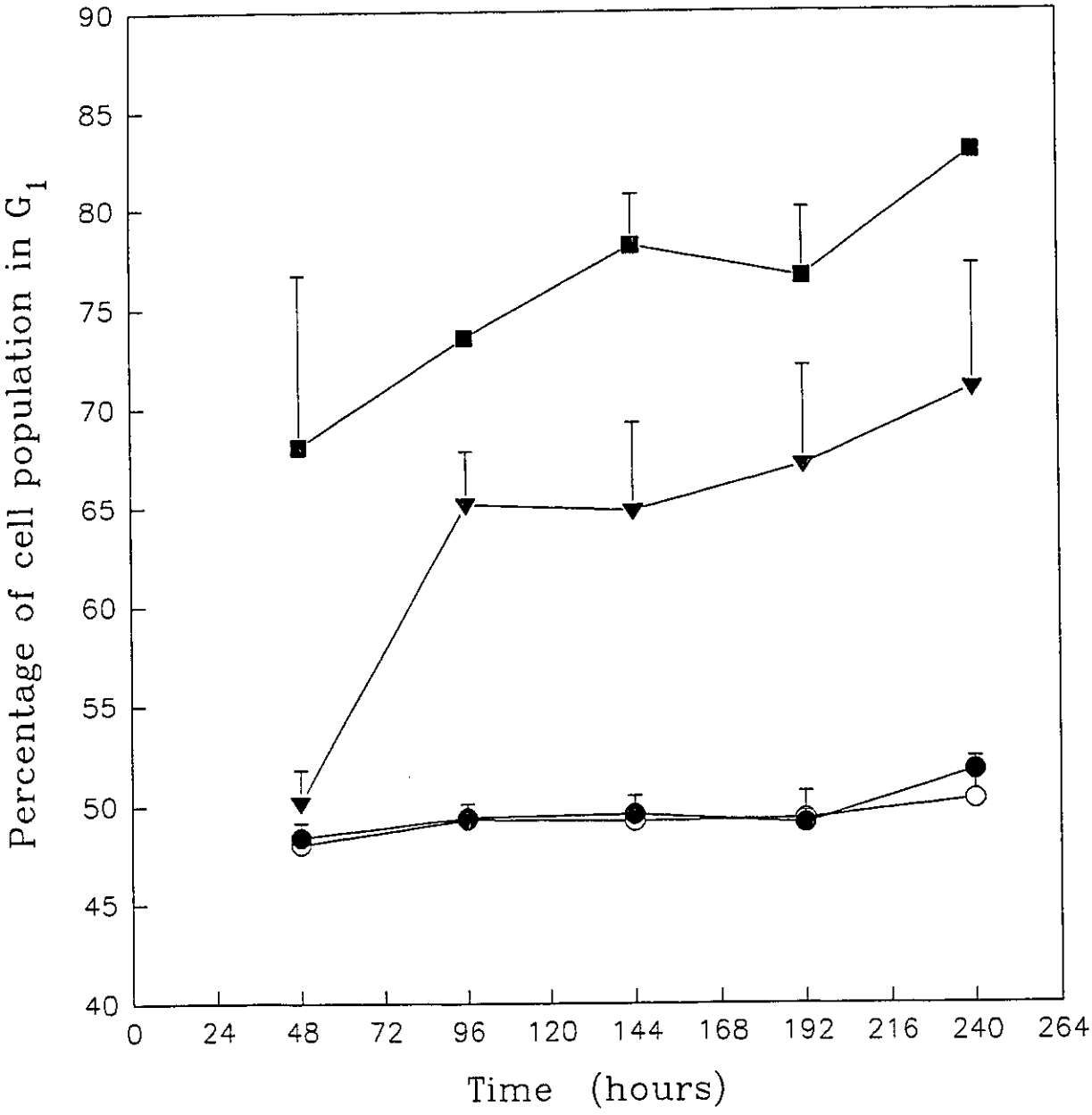
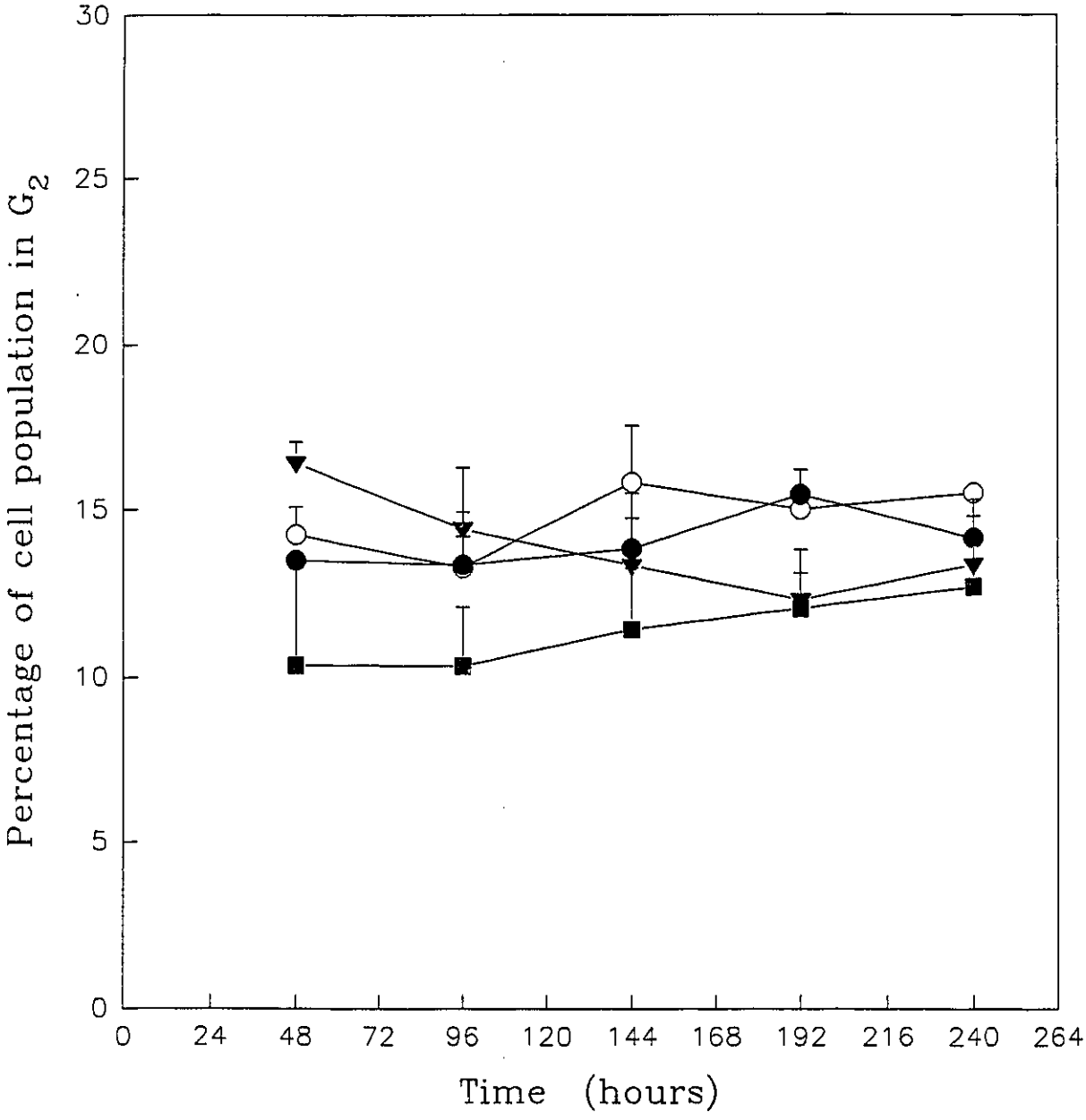

Percentage of cell population in G_1
90
85
80
75
70
65
60
55
50
45
40
0
24
48
72
96
120
144
168
192
216
240
264
Time (hours)

Figure 20. Distribution of HL-60 cells in the G_2 phase of the cell cycle during induction of differentiation by 5 x 10^{-7} M vitamin D_3. The DNA content of individuals in treated and control populations was assessed by flow cytometry of propidium iodide-stained cells and the percentage of cells in G_2 phase determined using Multicycle, a standard cell cycle analysis software package. Results plotted are the mean of triplicate experiments. Error bars represent the standard error of the multiple measurements. Open circles indicate results for untreated HL-60 cells, filled circles designate results for ethanol-treated controls, filled triangles those of vitamin-treated cells which remained nonadherent and filled squares the results for vitamin-treated cells which became adherent.


Percentage of cell population in G_2
30
25
20
15
10
5
0
0
24
48
72
96
120
144
168
192
216
240
264
Time (hours)

in the treated cells which distinguishes them clearly from the controls. Induction of HL-60 with 5 x 10^{-7}M vitamin D_3 did not affect the proportion of cells in the G_2/M phase of the cell cycle.

The effects of TPA treatment on the fraction of HL-60 cells in the G_1 phase of the cell cycle are depicted in figure 21. The untreated HL-60 cells and the ethanol treated controls showed similar small variations throughout the course of treatment. At 4 hours 52% of these cells were found in G_1. A slight increase to 57% G_1 cells occurred from 12 to 24 hours, followed by a return to 52% of cells in G_1 during the final 24 hours of the treatment period. The error bars on these data points indicate that these slight changes were both consistent and significant. In the nonadherent subpopulation of TPA-treated cells, no difference from controls was noted up to the 12 hour time point. From 12 to 36 hours, the fraction of these cells in G_1 increased steadily to reach 71%, at which level it remained until 48 hours. In the adherent subpopulation, changes were more pronounced during the first half of the induction period. At 12 hours, 63% of these cells were in G_1. This fraction increased slowly and steadily to reach 71% G_1 cells at 36 hours. From 36 to 48 hours no further changes were seen. As indicated by the error bars, inter-experimental variation in the treated cell subpopulations was minimal. TPA treatment caused an accumulation of HL-60 cells in G_1, evident in the adherent subpopulation first, but affecting the nonadherent cells to the same extent after a short delay.

Figure 22 documents the effects of induction of HL-60 by 3 x 10^{-8}M TPA on the proportion of cells in the G_2/M phase of the cell cycle. Both the untreated HL-60 cells and the ethanol treated controls showed an apparent slight decline from 12% of cells in G_2 during the first 24 hours of the induction period to 9-10% at the 36 and 48 hour time points. The error bars on these data points indicate that this trend was not significant. The nonadherent TPA-

Figure 21. Distribution of HL-60 cells in the G_1 phase of the cell cycle during induction of differentiation by 3 x 10^{-8} M TPA. The DNA content of individuals in treated and control populations was assessed by flow cytometry of propidium iodide-stained cells and the percentage of cells in G_1 phase determined using Multicycle, a standard cell cycle analysis software package. Results plotted are the mean of triplicate experiments. Error bars represent the standard error of the multiple measurements. Open circles indicate results for untreated HL-60 cells, filled circles designate results for ethanol-treated controls, filled triangles those of TPA-treated cells which remained nonadherent and filled squares the results for TPA-treated cells which became adherent.

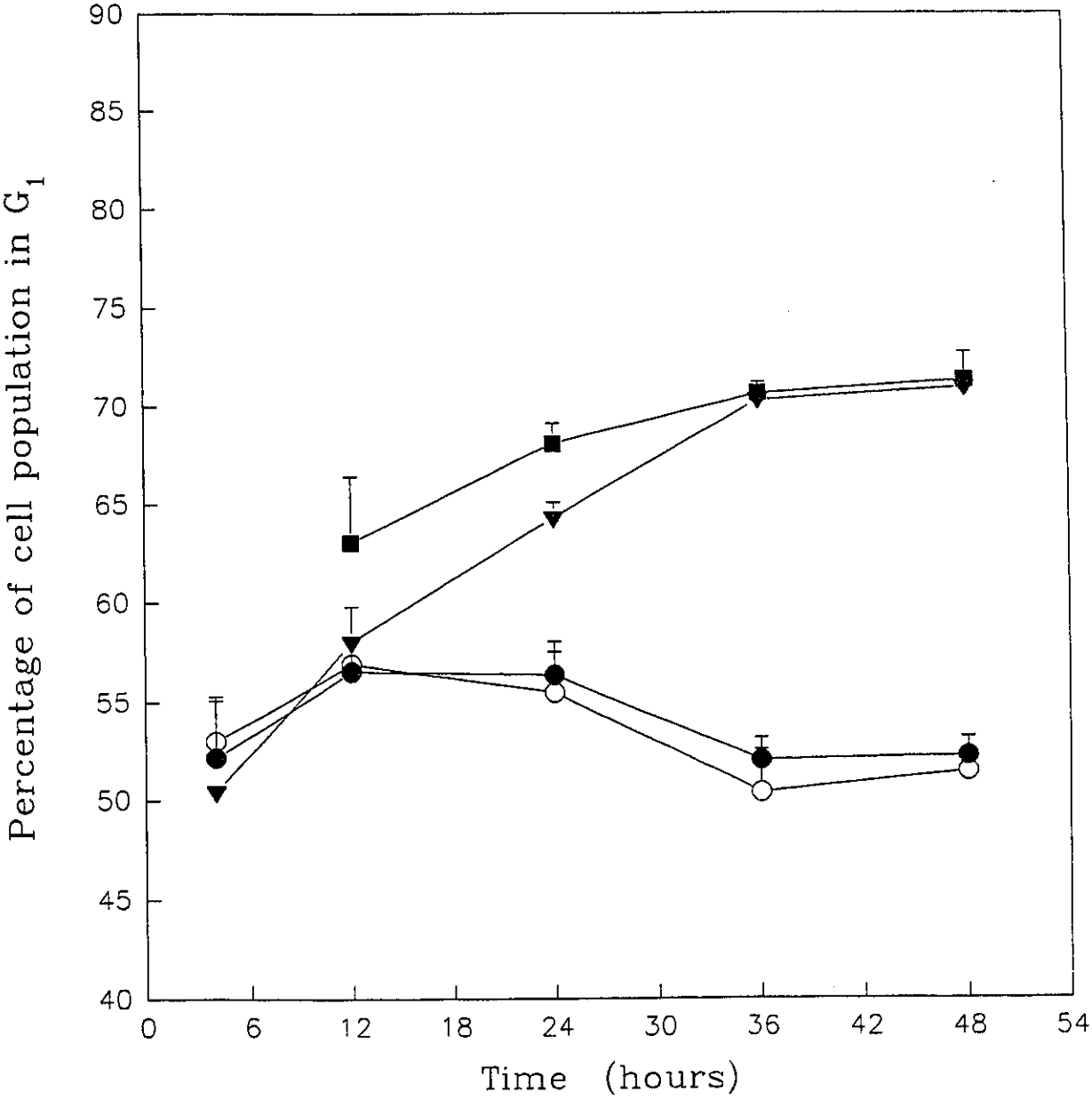
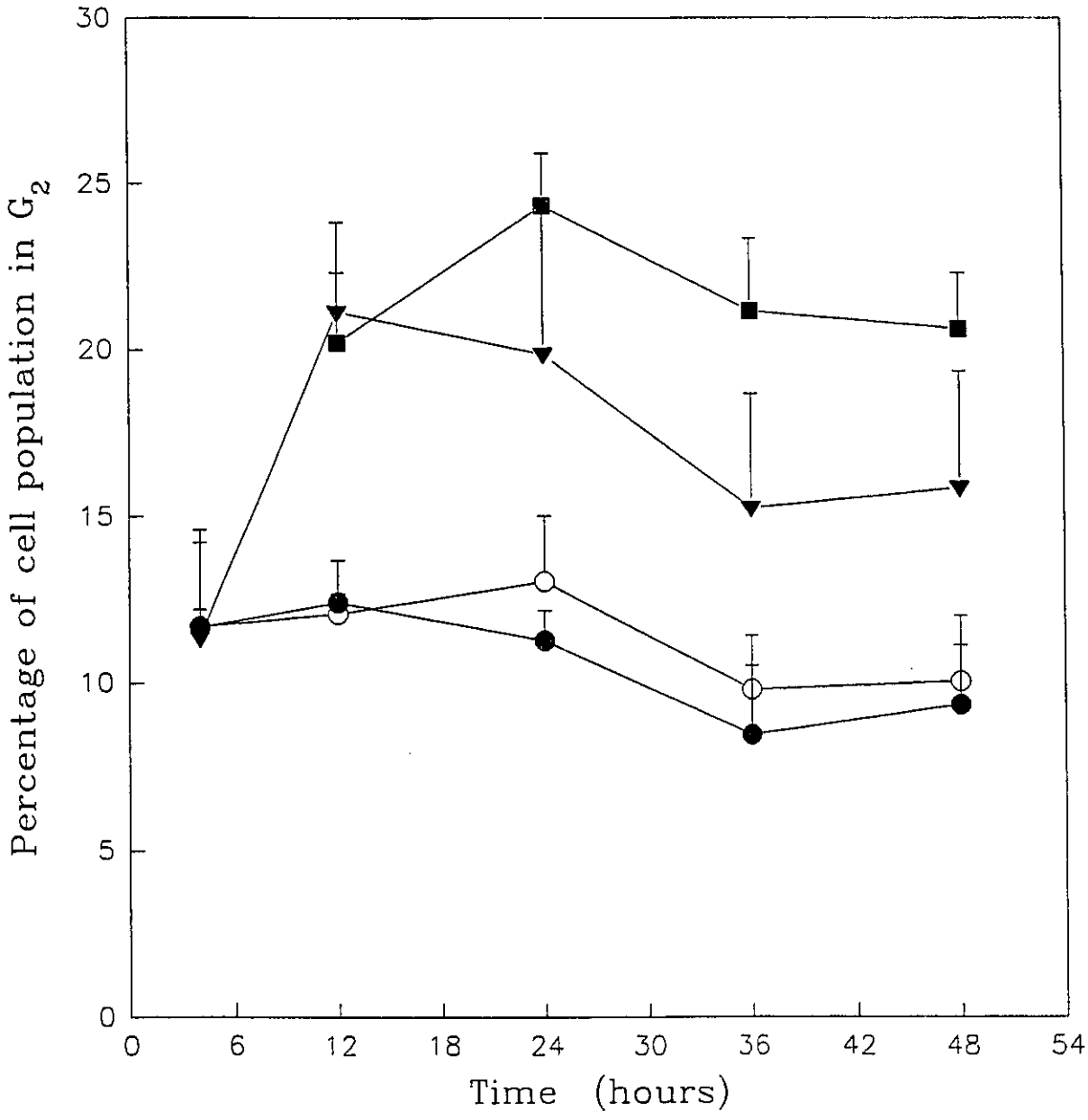

Percentage of cell population in G_1
90
85
80
75
70
65
60
55
50
45
40
0
6
12
18
24
30
36
42
48
54
Time (hours)

Figure 22. Distribution of HL-60 cells in the G_2 phase of the cell cycle during induction of differentiation by 3 x 10^{-8} M TPA. The DNA content of individuals in treated and control populations was assessed by flow cytometry of propidium iodide-stained cells and the percentage of cells in G_2 phase determined using Multicycle, a standard cell cycle analysis software package. Results plotted are the mean of triplicate experiments. Error bars represent the standard error of the multiple measurements. Open circles indicate results for untreated HL-60 cells, filled circles designate results for ethanol-treated controls, filled triangles those of TPA-treated cells which remained nonadherent and filled squares the results for TPA-treated cells which became adherent.


Percentage of cell population in G_2
30
25
20
15
10
5
0
0
6
12
18
24
30
36
42
48
54
Time (hours)

treated cells diverged from the controls after 4 hours, the fraction of cells in G_2 rising to 21% by 12 hours. From 12 to 36 hours this percentage decreased steadily to reach 16% G_2 cells, after which it did not change further. The adherent treated subpopulation also showed an increase in the proportion of cells in G_2/M to 21% at 12 hours. This fraction then rose slightly to peak at 24% G_2 cells at 24 hours. From 24 to 48 hours, the proportion of G_2 cells returned gradually to the 21% mark. The error bars on the data points indicate that the slight variations seen from 12 to 48 hours in the treated cells were not significant. TPA treatment gave rise to an increase in the proportion of HL-60 cells in G_2. This effect was more pronounced and sustained in the adherent cell subpopulation, while the cells that remained in suspension displayed a maximal percentage of G_2 cells after 12 hours of treatment.

DISCUSSION

EXPRESSION OF LAMINS A AND C IN DIFFERENTIATING HL-60 CELLS

The expression of nuclear lamins A and C in HL-60 cells induced to differentiate under the influence of two inducers of the monocyte/macrophage path was found to differ with the cell treatment. HL-60 cells exposed to 5 x 10^{-7}M vitamin D_3 did not express these lamin proteins at a level significantly different from untreated populations during the course of repeated 10 day inductions (figure 3). In dramatic contrast, HL-60 treated with 3 x 10^{-8}M TPA expressed lamins A and C in the vast majority of cells after 24 hours of incubation with the drug (figure 4).

This startling observation raised a number of new questions in addition to the original query which prompted the thesis research. The project began with the question of whether lamins A and C expression was associated with expression of the differentiated phenotype. In addition, it was hoped that examination of lamins A/C expression in the temporal context of expression of other markers of differentiation could provide clues to lamin function. The discovery of differentiation in the HL-60 cell system without the concomitant expression of lamins A and C protein led to additional enquiries. First, it raised the need to confirm that differentiation was occurring in the presence of both TPA and vitamin D_3. It also provoked a closer examination of the cells in their post-treatment states. Did the same cell types arise after treatment with the two inducers? How did the resultant cell types compare to macrophages?

After re-examining the treated HL-60 cells in the context of these questions, what could be deduced about the expression of lamins A and C and the differentiation process? How did lamins A/C expression patterns compare to those of vimentin, the cytoplasmic intermediate filament protein in these cells? How did the observed patterns of lamins A and C expression compare with other studies of lamin expression? Taken together, could these results shed some light on the possible roles of lamins A/C ?

These questions were addressed experimentally by examining cell growth and a number of markers of differentiation in the treated cells, as described in Results. Perusal of the literature pertaining to lamin expression provided a broader context for interpretation of the data generated.

HL-60 CELL DIFFERENTIATION IN RESPONSE TO VITAMIN D_3 AND TPA

In order to confirm that the treated cells responded to either TPA or vitamin D_3 by engaging a program of differentiation, several characteristics of this process were assessed experimentally. Growth arrest of the HL-60 cells, a hallmark of differentiationin this system, was examined using several approaches. Cell surface markers were also studied to determine whether the expression of the monocyte-specific antigen 63D3 and of TR changed under the influence of the inducers.

A) HL-60 Cell Response to Vitamin D_3 Treatment

The HL-60 cell response to treatment with vitamin D_3 was characterised by growth arrest of the cells which became adherent, partial growth inhibition of the cells which remained in suspension and dramatic changes in the markers of differentiation studied.

In response to induction by 5 x 10^{-7}M vitamin D_3, distinct effects on cell growth were noted in the adherent and nonadherent subpopulations. Looking at the population as a whole, cell density stabilised after 120 hours of treatment (figure 13). This time frame is consistent with that seen in several early studies of vitamin D_3 differentiation which monitored population growth (Miyaura et al., 1981; Murao et al., 1983; and Inaba et al., 1986).

Cellular replication in the adherent subpopulation was found to be completely inhibited by both methods employed to assess proliferation. Cells pulse-labelled with ^{3}H-thymidine demonstrated almost complete inhibition of DNA synthesis, an effect evident by 48 hours and maximal after 96 hours of treatment (figure 15). Cell cycle analysis demonstrated a steady decline in the proportion of cells in S phase, apparent within 48 hours of treatment (figure 17). The proportion of cells found in the G_1 phase of the cell cycle rose throughout the differentiation period at rates that mirrored the decrease of cells in S (figure 19). Inhibition of DNA synthesis seen in the adherent treated cells could be attributed to a vitamin-induced block of the cell cycle in G_1. This block was evident after 48 hours and remained effective throughout the treatment period. These results confirm and extend a previous report of a vitamin-induced cell cycle block in G_1 seen at 48 hours of treatment in the adherent subpopulation (Bar-Shavit et al., 1986).

Cellular replication in the nonadherent subpopulation was found by the above methods to be only partially inhibited. These cells had ^{3}H-thymidine incorporation levels similar to controls throughout the first 96 hours of treatment (figure 15), with a decrease to 50% of control

levels seen during the last half of the induction period. Cell cycle analysis confirmed this result. The nonadherent subpopulation displayed a drop in the proportion of cells in S phase only after 96 hours (figure 17), while the proportion of cells in G_1 rose during the induction period in a pattern that mirrored the trends seen in the S phase cells (figure 19). As seen in the adherent subpopulation, the decrease of nonadherent treated cells in S phase was due to a vitamin-induced cell cycle block in G_1. In the latter case the block did not take effect until the fourth day of treatment and did not affect the entire subpopulation; a fraction of the cells continued to cycle. Once again, this result matches that of Bar-Shavit and colleagues (1986), who found using flow cytometry that vitamin D_3 only partially inhibited the growth of those cells which remained in suspension.

Further insight into the effects of vitamin D_3 on HL-60 cells was gained by examining the results of immunostaining for differentiation-related markers. Staining for the monocyte-specific protein recognised by the 63D3 antibody, depicted in Figure 8, revealed that the expression of this marker was enhanced in all treated cells. 63D3 expression began to increase after 24 hours of exposure to the vitamin, and by 96 hours of treatment was detected in virtually all cells. This result concurs with the work of the Bar-Shavit group, although the kinetics of 63D3 expression was accelerated slightly in their hands; they found maximal rates of expression beginning at 72 hours (Bar-Shavit et al., 1983, 1986). This result suggests that vitamin D_3 treatment led to expression of at least partial monocyte phenotype. TR expression declined in all treated cells until the 120 hour time point (figure 11), a time frame coincident with the rise seen in 63D3 antigen expression. The subsequent rise in TR expression in treated cells could be interpreted as further progress along the differentiation path towards TR-positive

macrophages.

Taking into consideration both the cell growth and immunofluorescence profiles, it appears clear that treatment with 5 x 10^{-7}M vitamin D_3 does engage the process of differentiation in HL-60 cells which become adherent. This subpopulation showed a rapid response which included the differentiation hallmark of growth arrest and expression of monocyte-specific protein(s).

The response of the vitamin D_3-treated HL-60 cells which remained in suspension was less clear; growth arrest of these cells did not occur for a substantial period of time and did not affect the entire subpopulation. On the other hand, changes in marker expression identical to those seen in the adherent subpopulation were observed. It appears that vitamin D_3 treatment does induce expression of a differentiated phenotype in these cells but is unable to cause growth arrest of the entire subpopulation.

It appears that vitamin D_3 is not able to exert an overwhelming effect on the entire population of treated cells. To a certain extent this may reflect reality; as a potential actor in the differentiation of white blood cells *in vivo*, the vitamin would act in the context of a number of hormonal and other biochemical signals present in the immune system. Recreating the subtleties of this environment is a task beyond the limits of the culture system. On the other hand, the fact that HL-60 is a cell line capable of apparently indefinite propagation in culture may be significant to understanding its response to vitamin D_3. The transformation from its origin as a primary culture of myeloid leukemia cells to a stable cell line may well have involved a change in responsiveness to physiological agents which curtail growth. It is conceivable that HL-60 acquired the ability to grow in the presence of such agents, including vitamin D_3.

Finally, the response of HL-60 to vitamin D_3 may reflect the natural heterogeneity of the population in terms of its differentiated state. The immunostaining experiments performed for this thesis consistently showed background levels of lamins A/C, vimentin and 63D3 present in the untreated populations. At any given time, approximately 10% of the control populations were positive for these markers. These cells apparently differentiated "spontaneously" in culture, an observation is consistent with other published reports of HL-60 (Collins, Gallo and Gallagher, 1977; Paulin-Levasseur et al., 1989a). Given that a minority of cells in the population had differentiated, it is improbable that the remainder of the cells were all in one uniform, completely undifferentiated state. They more likely represented numerous, slightly different levels of "maturity" or plasticity. If so, it is conceivable that their response to a physiological inducer of differentiation, effective *in vivo* in specific temporal and biochemical context, would be mixed. Some of the population may well be partially unresponsive to such an inducer.

B) HL-60 Cell Response to TPA Treatment

An examination of the effects of TPA treatment on HL-60 cells revealed a rapid arrest of cell proliferation and dramatic alterations in cell morphology, but only limited change in antigenic markers of differentiation.

TPA-induced arrest of cell proliferation was consistently detected by all three methods employed to assess the replicative status of the cells. Haemocytometer counts (figure 14) demonstrated that the TPA-treated population did not increase but had a decline of living cells, evident at 36 hours. Death of TPA-treated HL-60 has been noted by previous investigators and

occurred in a similar time frame (Rovera, Santoli and Damsky, 1979; Murao et al., 1983 and Paulin-Levasseur and Julien, 1992). Figure 16 depicts the rapid suppression of ^{3}H-thymidine incorporation in TPA-treated cells. This effect was apparent by 8 hours and maximal after 24 hours, a time frame shorter than one cell cycle of the untreated HL-60. Once again, this result concurs with previous documentation of the extent and kinetics of inhibition of replication seen in this system (Rovera, O'Brien and Diamond, 1979; Paulin-Levasseur and Julien, 1992). Cell cycle analysis showed a decline in the proportion of treated cells in S phase , which reached a minimum at 24 hours (figure 18). The percentage of TPA-treated HL-60 in G_1 phase rose during the induction period, reaching a maximum by 36 hours (figure 21). This was significantly longer than the 24 hour time period required for the percentage of cells in S to drop to its minimum value. The discrepancy was accounted for by treated cells in the G_2 phase of the cell cycle. Exposure to TPA caused an increase in cells in G_2 which peaked at the 24 hour time point (figure 22). This G_2 block accounted for the quantitative and temporal differences seen between the decrease of cells in S and the accumulation of cells in G_1. The fraction of cells in G_1 was not large enough to account for the entire drop in S phase cells by itself; some of the decline was due to cells that remained in G_2. At 24 hours, when the G_2 block was maximal and the G_1 block quite significant, the fraction of S phase cells was the same as at 36 hours, when the percentage of cells in G_1 had increased slightly and the percentage in G_2 decreased slightly. In TPA-treated HL-60 cells complete inhibition of replication was seen within 24 hours of exposure to the drug. This phenomenon was due to two TPA-induced cell cycle blocks; most of the cells accumulated in G_1 phase with a smaller fraction arresting in G_2.

Treating HL-60 cells with 3 x 10^{-8}M TPA led to drastic changes in cell morphology

(figures 1 and 2). The vast majority of cells became adherent and elongated, with abundant cytoplasmic granules and lobed nuclei. Those that remained in suspension did not undergo these dramatic changes, but did show subtle alteration of the nuclear shape and appeared larger. Clearly, TPA treatment of HL-60 gave rise to cells that were morphologically distinct from controls. The morphology of the adherent subpopulation resembled that of isolated murine peritoneal macrophages to a great extent, as exemplified in figures 1K and 1M.

Further insight into the status of TPA-treated cells was sought by immunofluorescence staining for markers of differentiation. Throughout the treatment period, TPA-induced HL-60 cells did not express the monocyte marker 63D3 (figure 9). 63D3 is reported to be an early marker of differentiation along the monocyte/macrophage pathway (Nunez et al, 1982), as was found in vitamin D_3-treated HL-60. The fact that 63D3 is never expressed by the TPA-treated subpopulations suggests that this drug does not induce the first steps of the monocytic path. The expression of TR in TPA-treated HL-60 cells was completely downregulated within 4 hours (figure 12). This drop in TR expression could be interpreted as commitment of the TPA-treated cells to the monocyte pathway, since monocytes are TR-negative (Andreesen et al, 1986). However , the concurrent rise in 63D3 expression expected if this were true was never seen. TPA-treated HL-60 showed no signs of becoming TR-positive during the remainder of the induction period.

On the bases of morphological change and arrest of growth, it is clear that TPA treatment of HL-60 cells leads to an alternate, differentiated state. The dramatic phenotypic changes seen in the majority of the treated population indicated that this was a novel state. At the very least it involved marked changes from the untreated population in the cytoskeletal components

involved in establishing an adherent, elongated shape, in the proteins present in the newly abundant cytoplasmic granules and in TR expression.

Two aspects of the changes observed in TPA-treated HL-60 cells merit further discussion: the kinetics of TR downregulation and the induction of a G_2 cell cycle block.

During TPA induction of HL-60 TR expression declined by 4 hours, while growth was not fully inhibited until 12 hours (figures 12 and 16). This raises the question of whether TR expression is a marker of the differentiated state or of lineage-independent growth inhibition. The increase in TR expression in macrophages is a characteristic of the differentiated phenotype, as iron uptake and storage is a function of these cells (Andreesen et al., 1984). However, it appears that the particulary rapid decrease in TR expression seen upon induction of differentiation by TPA may be related to cessation of growth. Ho and colleagues (1989) demonstrated by Northern blotting that TR mRNA in TPA-induced HL-60 does not decrease significantly until 12 hours of treatment. Thus, the initial decrease in TR expression in these cells is not due to transcriptional downregulation, which is effective only at later time points. Given that the drop in TR expression preceded growth arrest and the differentiation-associated change in expression of the TR gene, the authors argued that changes in TR protein upon exposure to TPA reflected a role for the protein in the cessation of growth. Another study soon followed which demonstrated that the initial drop in TR protein in this system was due to internalisation of the receptor (Trayner and Clemens, 1990). This change would not have been detected by the TR staining protocol used in this thesis as the cells were not permeabilized before incubation with the primary antibody. The authors also demonstrated that blocking transferrin uptake by the HL-60 cells resulted in inhibition of proliferation without

differentiation. This strongly suggests that the early drop in TR seen upon exposure to TPA is partially responsible for cessation of proliferation, as it decreases the availability of transferrin needed for growth.

TPA treatment of HL-60 resulted in a minor cell cycle block in G_2. This effect was unexpected. Perusal of the literature revealed that induction of a G_2 block is a general feature of TPA treatment not confined to HL-60 cells. A minor G_2 cell cycle block, maximal at early time points has been noted in the inducible haematopoietic cell lines KOPM-28 (Tsuda et al., 1988) and MPC-11 (Giese, Kubbies and Traub, 1990) and in non-inducible HeLa cells (Kinzel, Bonheim and Richards, 1988). The latter group used time-lapse photography to examine the G_2 block and determined that it affected cells before visible prophase. Tsuda and coworkers (1988) used synchronised cell cultures to determine that the G_2 block acted on some, but not all of the cells in S and G_2 at the time of inducer exposure.

HL-60 CELL PHENOTYPE AFTER TREATMENT WITH VITAMIN D_3 AND TPA

As is clear from the above discussion of differentiation in HL-60, the final phenotype of the cells after induction by Vitamin D_3 differs from those that were exposed to TPA. While neither was identical to the phenotype of the mouse peritoneal macrophages used as controls, TPA-treated HL-60 were the most similar.

A) HL-60 Cell Phenotype Following Treatment with Vitamin D_3

Vitamin D_3 treatment of the HL-60 cells led to an immediate drop in TR expression and an increase in the expression of the monocyte marker protein recognised by the 63D3 antibody (figures 8 and 11). Some cells adhered to the substratum and ceased replication (figures 1, 2, 15 and 17). In the last half of the induction period, replication was also inhibited in some of the nonadherent subpopulation (figures 15 and 17) and the level of TR expression in all cells rose (figure 11). Throughout these changes, the expression of lamins A and C and of vimentin did not differ significantly from controls (figures 3 and 5). As documented in the literature, vitamin D_3-treated HL-60 express other surface antigens specific to monocyte/macrophage, including OMK-1 on virtually all cells (Murao et al., 1983), CD14 on 80% of cells (Fischkoff and Rossi, 1990) and CD3 on 50% of cells (Miyaura et al., 1981). Lineage-specific enzyme activity is also induced; non-specific esterase induction is seen in 50-80% of vitamin-treated cells (Bar-Shavit et al., 1983; Murao et al., 1983; Inaba et al., 1986; Botilsrud et al., 1990).

A comparison of the HL-60 cells following ten days of vitamin D_3 treatment with murine macrophages reveals a multitude of differences between the two. They had distinct morphologies (figures 1 and 2) and a comparison of Table 1 with figures 5 and 8 shows that their profiles of vimentin and 63D3 expression were completely contradictory. As seen in figure 2 and table 1, lamins A/C expression was not induced in the vitamin-treated HL-60, while time in tissue culture did stimulate lamins A and C expression in the macrophages. The only measured parameter in which these two cell types displayed some similarity was the expression of TR protein; the vast majority of macrophages were TR-positive (table 1) and approximately 50% of the vitamin-treated cells finished the induction period TR-positive (figure 11). Moreover, the pattern of staining was similar in both cell types; a sparse scattering of small

patches on the cell surface was seen in positive cells (figures 10F and 10L). Clearly, vitamin D_3-treated HL-60 cells do not resemble macrophages to any great extent, despite a similarity in their expression of TR protein.

What then is the state of the cells following exposure to vitamin D_3? The results of staining for the monocyte marker 63D3 indicate that the cells were engaged on the monocytic path and remained so throughout the treatment period. The results of staining for TR suggest that the cells were capable of expressing some protein(s) typical of macrophages, although to a more limited extent. These indications are consistent with the literature record of marker and enzyme expression discussed above. The phenotype of the treated cells, which remained small and round even when adherent, was more suggestive of monocytes than macrophages. The cells appear to be intermediates on the monocyte/macrophage development path which resemble monocytes but may have differentiated far enough to begin to express some macrophage protein(s). This conclusion is supported by a report of analysis of the two dimensional gel electrophoresis protein profile of differentiated HL-60 and blood monocytes (Murao et al., 1983). Neither TPA- nor vitamin D_3-treated HL-60 had a pattern of protein expression identical to monocytes, but that of the vitamin D_3-induced cells was very similar.

B) HL-60 Cell Phenotype Following Treatment with TPA

Treatment of HL-60 cells with 3×10^{-8} M TPA led to an immediate drop in expression of TR protein (figure 12) and arrest of cell growth within 24 hours (figures 16 and 18). The majority of cells became adherent and underwent dramatic changes in morphology, becoming strikingly similar to macrophages (figures 1K and 2K). Expression of the monocyte marker

63D3 was not induced (figure 9). The expression of vimentin protein rose throughout the first 36 hours of the treatment period, then declined slightly (figure 6). Lamins A and C were expressed in response to TPA in the vast majority of the treated cells, their level of expression rising throughout the treatment period (figure 4). Expression of differentiation-associated enzyme activity is well documented in TPA-treated HL-60; acid phosphatase, non-specific esterase, and lysozyme activity have all been recorded (Rovera, Santoli and Damsky, 1979; Murao et al., 1983). TPA-treated cells are also phagocytic (Rovera, O'Brien and Diamond, 1979). Finally, downregulation of the cell cycle regulatory genes cdc2, cdc25, cyclinA and cyclinB has also been reported (Hass et al., 1992).

Comparison of the 24 hour to 48 hour TPA-treated HL-60 cell samples and mouse peritoneal macrophages reveals many similarities between the two. Their morphologies were strikingly similar, as exemplified in figures 1K and 1M. Both cell types expressed vimentin protein and did not express the monocyte marker protein 63D3 (compare figures 6 and 9 with table 1). TPA treatment of HL-60 induced expression of lamins A and C proteins (figure 4), while macrophages were induced to express these proteins by incubation in culture (table 1). The sole phenotypic marker studied which differed between these two populations was TR expression. TPA-treated HL-60 did not express this marker (figure 12), while the majority of macrophages did (table 1). This difference may reflect the fact that TPA causes cell death within a short time of exposure. It might interfere with expression of genes necessary for the growth and maintenance of the cells, a category which could include the TR gene (Trayner and Clemens, 1990).

Clearly, the state induced by TPA treatment of HL-60 is very macrophage-like,

particularly in terms of cell size, shape and IFP content.

EXPRESSION OF LAMINS A AND C IN THE CONTEXT OF HL-60 CELL DIFFERENTIATION

During the course of thesis research, it was determined that induction of differentiation in HL-60 cells by 5 x 10^{-7}M vitamin D_3 did not result in expression of lamins A and C, while treatment with 3 x 10^{-8}M TPA effectively induced these proteins. As discussed above, although both inducers affect the monocyte/macrophage path, they differ in the end product generated. An examination of the patterns of lamins A and C expression in the context of these differences could provide insight into their place in the developmental program.

A) Lamins A/C Expression is Not Associated With Inhibition of Replication

Induction of HL-60 cells by vitamin D_3 clearly demonstrated that lamins A/C expression was not linked to the cessation of growth. In these experiments, growth arrest of the treated cells occurred by 48 hours in the adherent subpopulation and by 96 hours in approximately 50% of the nonadherent subpopulation (figures 15 and 17). Throughout the entire induction period, no increase in lamins A and C expression was seen (figure 3). This conclusion was supported by the data obtained from the TPA treatment experiments. A comparison of figures 16 and 4 shows that in the adherent subpopulation, DNA replication was completely inhibited by the 12 hour time point, while lamins A and C expression had increased to only half its maximum point.

The timing of these two changes was not coincidental; induction of lamins A and C expression followed inhibition of replication. This result is consistent with a previous study of lamins A and C expression in HL-60 cells treated with TPA (Paulin-Levasseur and Julien, 1992).

The total dissociation of lamins A and C expression from arrest of growth in the vitamin D_3-treated cells provided a welcome clarity in its confirmation of the previous and current results for TPA-treated HL-60. The short time frame of induction in the TPA-treated cells can make it a challenge to decipher the relative appearance of differentiation-associated changes accurately, as can be seen in comparing the results for lamins A/C expression and inhibition of replication in the nonadherent subpopulation (figures 4 and 16). The expression of lamins A and C protein does appear to lag behind the inhibition of growth for these cells as well, but the temporal differences in this case are very slight. In addition, the consistency of this result for both treatments argues that it is a general phenomenon of HL-60 differentiation towards monocytes/macrophages and not an inducer-specific effect.

B) Lamins A/C Expression is Not Associated With Expression of the Differentiated Phenotype.

Under the influence of both inducers of HL-60 cells employed for this thesis it was found that lamins A and C expression was not associated with the markers of differentiation studied. In vitamin D_3-treated HL-60, expression of the monocyte marker 63D3 was induced in virtually all the treated cells, and TR expression was first decreased then returned to near control levels during the course of treatment (figures 8 and 11). While the morphology of the vitamin-treated cells did not change completely, a subpopulation did become adherent and changes in nuclear

shape and prominence of the nucleoli were commonly noted (figures 1 and 2). These changes all occurred in the absence of lamins A and C expression (figure 3). Following TPA treatment, expression of TR protein decreased within 4 hours (figure 12). Large, adherent cells with dramatically altered morphology also appeared within 8 hours (figures 1 and 2). Yet by the 12 hour time point, lamins A and C expression in these changed cell populations was only induced to half its maximal level (figure 4); it was not concurrent with any of the phenotypic changes.

It is possible to attribute to chance the fact that changes in expression of both surface markers of differentiation chosen for this study preceded change in lamins A/C expression. This argument is strongest in the case of the monocyte marker 63D3, which proved to be a very early indicator of change in the vitamin D_3-treated HL-60 cells, preceding growth arrest in the nonadherent subpopulation (figures 8 and 15). However, the changes observed in TR expression and cell morphology do not lend support to this argument. In the cells induced to differentiate with vitamin D_3, TR expression rose to near control level during the last half of the induction period (figure 11). This rise in TR expression was a late event in the differentiation process, beginning four days after the rise in 63D3 expression and inhibition of growth in the adherent subpopulation were noticed (figures 8 and 15). Lamins A and C expression was not linked with even this very late change in expression of the differentiated phenotype (figure 3). In addition, morphological changes in the treated cells were seen prior to or in the absence of lamins A/C expression for both inducers, as noted above. Expression of these lamins was not part of the myriad of differentiation-associated changes necessary to bring about the alterations observed in cell shape and adherence.

C) Lamins A/C Expression May Be a Post-Differentiation Event

Data obtained from both the treated HL-60 cells and the murine macrophages indicated that the expression of lamins A and C is a very late or post-differentiation event that follows expression of the differentiated phenotype. Vitamin D_3-treated HL-60 cells never expressed lamins A and C (figure 3) despite growth arrest of the bulk of the population (figures 15 and 17) and their changes in expression of markers of differentiation (figures 8 and 11). In the TPA-treated HL-60 cells which became adherent, inhibition of replication, dramatic morphological alterations and drop in the expression of TR all occurred before maximum expression of lamins A/C (figures 16,1,2,12 and 4). These indicators all reached maximal points at or before the 12 hour mark, while lamins A and C expression continued to increase from 24 to 48 hours. Newly-isolated murine macrophage cells were largely lamins A/C-negative, while those maintained in culture for a week were largely positive (table 1). In these cells, the expression of lamins A and C proteins was not characteristic of lineage so much as metabolic state. These results confirm trends documented in cells and cell lines of haematopoietic origin by Röber and colleagues (1990a, 1990b) which will be discussed in more detail below.

The myelocytic lineage retains enough developmental plasticity for monocytes to become macrophages and macrophages to become activated given the appropriate signals. The activated macrophage thus represents a final possible transformation. Based on the above results, lamins A and C expression is seen only upon loss of all plasticity. Expression of lamins A/C does not appear to be linked to any of several differentiated phenotypes possible for these cells, but appears upon the cells' loss of ability to alter their phenotype in response to their environment.

EXPRESSION OF LAMINS A AND C IN THE CONTEXT OF IFP EXPRESSION

Throughout the course of HL-60 cell differentiation induced by vitamin D_3 or TPA treatment, the expression of vimentin was examined in parallel to the expression of lamins A and C. Vimentin is the expected cytoplasmic IFP in these cells. It was hoped that a comparison of vimentin and lamins A/C expression could place the changes seen for lamins A and C into the broader context of IFP regulation in this system.

A) IFP Expression Following Treatment With Vitamin D_3

In HL-60 cells induced to differentiate with Vitamin D_3, there was no significant trend of change in the expression of either lamins A/C or vimentin (figures 3 and 4). These results do not permit a comparison of the kinetics of lamins A/C and vimentin expression. However, the conclusions drawn above for lamins A and C can also be applied to vimentin expression. Namely, in HL-60 cells treated with Vitamin D_3, expression of vimentin is not linked with the cessation of growth or expression of the differentiated phenotype, including the adherence of treated cells to the substratum.

B) IFP Expression Following Treatment With TPA

Lamins A/C and vimentin were detected in the vast majority of TPA-treated HL-60 cells, but the kinetics of their expression differed. Lamins A/C expression rose steadily from 8 to 24 hours of treatment, while the expression of vimentin increased at a rapid pace from 8 to 12

hours and then at a slower rate through to the 36 hour time point (figures 4 and 6). This suggests that the differentiation events occurring in the 4-12 hour time frame, such as maximal inhibition of growth in the adherent cells (figure 16) were closely associated with vimentin expression. While lamins A and C also began to be expressed during this period, their induction was not particularly strong at the early time points. After the 36 hour time point, the expression of lamins A/C remained steady in the nonadherent subpopulation and rose slightly in the adherent subpopulation. The decline in vimentin expression during this period indicated that it is not as closely associated with very late or post-differentiation events as is the expression of lamins A/C. Taken together with the preceding discussion of the temporal context of lamins A and C expression, these results suggest that vimentin expression is a "middle" event in the differentiation process in HL-60, appearing quickly upon maximal inhibition of growth. It is not as necessary in the very late or post-differentiation stages. These results confirm a previous study which examined the expression of IFP in the context of TPA-induced HL-60 differentiation (Paulin-Levasseur and Julien, 1992). Lamins A and C were not as closely associated as vimentin with the arrest of growth. Their expression peaked with the very late or post-differentiation events occurring in the 36 to 48 hour time range.

The kinetics of vimentin expression in HL-60 cells treated with TPA introduced an apparent contradiction to the results obtained with vitamin D_3-induced cells. The latter showed no association of vimentin expression with the differentiated phenotype, specifically no strong induction of vimentin after maximal inhibition of growth. Differences in treated cell morphologies suggest two possible explanations for this discrepancy. TPA-treated cells are significantly bigger than untreated and vitamin-induced HL-60, with a markedly lower

nucleus/cytoplasm ratio (figures 1 and 2). It is possible that the smaller untreated and vitamin-treated HL-60, with their limited cytoplasmic compartment, may not require expression of IFP for the general cytoskeletal functions of maintaining cell shape and cytoplasmic order. The constitutively-expressed actin filaments and microtubules present in the cells may be sufficient for these tasks below a critical size or nucleus/cytoplasm ratio. Thus even the vitamin D_3-treated cells which became adherent may not have exceeded the critical size/composition parameter(s) which necessitate the expression of vimentin. This argument is less convincing in the case of the nonadherent TPA-treated cells, which were vimentin positive but only slightly bigger than the adherent vitamin D_3-treated cells. A second explanation for the observed discrepancy may lie in the details of the different phenotypes produced by the two treatments. Vimentin expression in HL-60 may be associated primarily with specific characteristic(s) which differ after treatment with the two inducers. For example, differences in the abundance or distribution of vesicles following the two treatments may require expression of vimentin in the TPA-induced HL-60 for effective organization or support of these organelles.

EXPRESSION OF LAMINS A AND C DURING DIFFERENTIATION IN OTHER SYSTEMS

During the last four years, numerous studies probing the association of lamins A and C with differentiation have been published. As presented in the introduction, strong evidence linking the expression of lamins A/C with the differentiated state in haematopoietic cells and cell

lines emerged during the late 1980's. Later studies have confirmed this trend and for the most part have found evidence that lamins A/C expression is a late event in the differentiation process. The literature is not without conflict, however; reports of lamins A/C expression in undifferentiated HL-60 and of early rearrangement of these proteins in response to TPA-induced differentiation have also been published.

A) Lamins A and C Expression Appears to be a Late Event in Differentiation in Many Systems.

As described in the introduction, association of lamins A and C expression with the differentiated state in cells and cell lines of haematopoietic lineage was reported throughout the late 1980's. The absence of lamins A and C in cell lines representative of numerous stages of haematopoietic differentiation, in primary cultures of immature lymphocytes and in haematopoietic cells isolated from spleen, thymus, blood and bone marrow was established using IFM and immunoblotting (Paulin-Levasseur et al., 1988; Guilly et al., 1990; Röber et al., 1990a). Lamins A and C were detected in the HL-60 cell line after differentiation induced by TPA or DMSO and in macrophages produced by *in vitro* culture of monocytes or *in vivo* intraperitoneal stimulation by thioglycollate (Paulin-Levasseur et al., 1989a; Röber et al., 1990b). The association of lamins A and C with the mature phenotype was controversial only in the case of peripheral blood lymphocytes. Guilly and colleagues (1990) found that both T and B lymphocytes had a full complement of lamins, while the group led by Röber (1990a) could detect only B lamins in these cell types. A third report on lamin expression in lymphocytes raises the possibility that this discrepancy may be due to conditions which stimulate the cells.

An examination of lamins A and C mRNA levels revealed no detectable transcripts in unstimulated peripheral blood lymphocytes but appearance of both in concavalin A-stimulated cells (Stadelman et al., 1990). This explanation provides a comfortable parallel with the expression of lamins A and C in macrophages; as detected during thesis research and published previously (Röber et al., 1990b), macrophages express these proteins only after environmental stimulation which exploits the remaining developmental plasticity of the cells.

In addition to establishing that lamins A and C were associated only with differentiated cells of the haematopoietic lineage, several of these studies provided evidence indicating that lamins A/C expression was a very late or post-differentiation event. In their study of lamins A and C expression in macrophages, Röber and coworkers (1990b) found that only stimulated macrophages expressed these proteins. Monocytes and unstimulated macrophages were lamins A/C-negative. Results of this thesis concur completely, with the exception of a small minority of freshly-isolated macrophages which were lamins A/C positive. This could be due to partial stimulation of the cells during the 24 hour incubation period preceding my first staining or to partial intraperitoneal stimulation by the agent injected to increase macrophage yield. Despite this slight discrepancy, the trend of increased lamins A and C expression after stimulation was evident. Röber's group argue that in the case of macrophages, lamins A and C expression clearly occurs after commitment to a differentiation pathway; it is a post-differentiation event. Induction of these proteins appears to be related to loss of plasticity in the face of future environmental signals. They speculate that the lack of lamins A and C expression in cells of the haematopoietic system may generally reflect this association; because many of these cell types are capable of responding to stimuli, they retain some developmental plasticity despite their

differentiated phenotype and do not express lamins A and C. An earlier study by the same group (Röber et al., 1989) provided evidence of post-differentiation expression of lamins A and C in several tissues of the mouse embryo. In epidermal cells, lamins A and C expression appeared at gestational day 16 upon formation of the bilayered periderm and long after commitment of the cells to the epidermal lineage. In brain, lamins A and C expression was not seen until after birth. Neuronal cells are present at birth, but do no finish establishing connections with one another until some time later. Thus, neural tissue provides another clear example of post-differentiation expression of lamins A and C which appears to be linked to loss of plasticity.

A more recent study of lamins A and C expression in the embryonic carcinoma cell line P19 and F9 found results consistent with those described above and those of this thesis (Mattia et al., 1992). Induction of differentiation in these cell lines led to expression of lamins A and C, detectable at the RNA and protein levels. Kinetic analysis revealed that lamins A and C expression followed expression of the differentiated phenotype in these cells. The authors concluded that lamins A and C expression in this system is a late event, induced only after the cells were confined to a developmental program.

B) Lamin A Rearrangement Has Also Been Implicated In Early Events of HL-60 Differentiation

The literature is not without conflicting evidence of alternate expression patterns of lamins A and C proteins. Two recent publications have reported expression of lamins A and C in undifferentiated HL-60, with one study detecting change in lamin A distribution associated

with early events of differentiation in these cells.

Kaufmann (1992) used Northern and sensitive immunoblotting techniques to examine the lamin complement of HL-60. He found low levels of lamins A and C protein and RNA in undifferentiated HL-60 cells and in several non-inducible cell lines derived from leukemias of the myelocytic lineage. He argued that the presence of lamins A and C in the undifferentiated HL-60 samples was not attributable to the spontaneously differentiated minority of cells known to be present in such cultures (Collins, Gallo and Gallagher, 1977; Paulin-Levasseur et al., 1989a) since the same lamins were detected in related cell lines that were not inducible. Rather, he felt that results reflected a low level of lamin expression in all HL-60. However, no experimental evidence in his report directly confirms this assertion. Further, lamins A and C protein attributable to the minority of differentiated cells present in any untreated HL-60 cell culture is expressed in sufficient quantity to render it detectable by conventional blotting methods (Paulin-Levasseur et al., 1989a).

Another study focussing on lamin A reported the presence of this protein in undifferentiated HL-60 cells (Collard, Senecal and Raymond, 1992). Using IFM, the authors described lamin A present as a "cap" at one side of the nucleus in undifferentiated HL-60 cells. They detected redistribution of lamin A to the nuclear periphery within 7 hours of TPA induction. They attributed the novel detection of lamin A in untreated HL-60 to increased sensitivity of their antibody relative to that used in previous studies by Paulin-Levasseur and in this thesis. Having obtained some of this lamin A-specific antibody from Dr. Raymond, I was unable to repeat his findings in our HL-60 cells using either his protocol as published or my own. Untreated HL-60 cells were negative, TPA-treated HL-60 cells were positive for lamin

A. Thus, my experience does not support the claim of increased antibody sensitivity leading to novel detection of lamin A. It is possible that the HL-60 cells used by the group were lamins A/C positive before induction due to culture conditions. According to Dr. Raymond, his HL-60 are cultivated in a media without buffer, at a basic pH. It is published comment and my finding during thesis research that culture of HL-60 under basic conditions induces the cells (Fischkoff and Rossi, 1990) and results in expression of lamins A and C (Röber at al., 1990b). In response to TPA, the authors found that early rearrangements in lamin A distribution seen during differentiation were not dependent on protein synthesis; they involved only the lamin A already present. Further, the micrographs that document the nuclear localisation of the lamin A cap are not convincing. The lamin A cap is seen to lie outside the lamina as defined by lamin B, but it is not clear that it falls within the boundaries of the nucleus; it could be cytoplasmic. If so, the redistribution of these proteins upon induction of differentiation may represent their spreading throughout the expanding cytoplasmic compartment as the nucleus/cytoplasm ratio decreases. Indeed, the paper documents lamin A incorporation into the lamina only at 24 hours. Earlier time points all show a wide band of diffuse lamin A stain apparently exterior to the nucleus, where it occupies the same area as the vimentin filaments in double-stained cells. Cytoplasmic redistribution limited to existing lamin A is not likely to impact on the differentiation programme of HL-60 since the protein would not be in contact with the DNA. The temporal increase in newly synthesized lamin A detected during the differentiation period concurred with that seen by Paulin-Levasseur and Julien (1992) and in this thesis, peaking after 24 hours.

EXPRESSION OF IFP DURING DIFFERENTIATION IN OTHER SYSTEMS

Vimentin expression during differentiation has also been a topic of interest in the literature. Reports to date, including some of those described above , concur with the major trends seen in this thesis: vimentin expression was not coupled to the expression of lamins A and C and was closely associated with the cessation of growth.

In their survey of haematopoietic tissues, Röber's group (1990a) found that lamins A/C expression was not coupled with that of vimentin. Several of the cell types which lacked lamins A and C expressed vimentin. The kinetic study of TPA-induced HL-60 by Paulin-Levasseur and Julien (1992) discussed above showed independent response patterns for vimentin and lamins A and C, which were confirmed by this thesis. This study also examined the effects of TPA on vimentin expression in the plasmacytoma cell line MPC-11. These cells did not express lamins A and C after induction, providing further confirmation of the trend. Finally, a recent survey of numerous inducers of differentiation in HL-60 and U-937 cells revealed several that led to uncoupled expression of lamins A/C and vimentin, including retinoic acid in HL-60 cells (Rius et al., 1993). In cells and cell lines of haematopoietic lineage, expression of lamins A and C and cytoplasmic IFP is not coordinately regulated.

Vimentin expression in differentiating cells appears to be part of the expression of the differentiated phenotype, temporally associated with the cessation of growth. In their study of TPA-treated MPC-11 and HL-60 cells, Paulin Levasseur and Julien (1992) found that in both cell lines maximal expression of vimentin in the population was seen coincident with maximal

inhibition of growth. In HL-60 induced to differentiate towards granulocytes by retinoic acid, Leung et al. (1992) found that peak vimentin expression followed inhibition of growth very closely and preceded peak expression of other markers of differentiation. This pattern of vimentin expression during differentiation thus appears consistent across several haematopoietic cell lines. The association of vimentin with cessation of growth is not absolute; vitamin D_3-treated HL-60 in this thesis and haematopoietic cell types which are both vimentin and lamins A/C-negative (Röber et al., 1990a) demonstrate that replication can cease and differentiation proceed without expression of vimentin. Rather, when vimentin expression is part of the differentiated phenotype, it appears coincident with or closely following the inhibition of replication.

THE ROLE OF LAMINS A AND C IN DIFFERENTIATION

Defining the role(s) of lamins A and C expression during differentiation has also been the goal of several recent publications reporting on the developmentally "unscheduled" expression of lamins A and C proteins induced by transfection of lamins A/C-negative murine embryonic carcinoma cell lines. The first report of this experimental approach appeared in 1990. Collard and Raymond reported the transient transfection of human lamin A or C into P19 and F9 cells. They detected expression of the human proteins by IFM and observed that they were correctly incorporated into the existing lamina. Transfected cells which entered mitosis showed the normal pattern of solubilization of lamins A and C

followed by incorporation into the lamina of daughter cells in late telophase/early G_1. The authors conclude that the biochemical machinery involved in lamins A/C processing and dynamics is present in cells which do not normally express these proteins. A later publication by Peter and Nigg (1992) examined the effect of unscheduled lamin A expression on the differentiation state and potential of embryonic carcinoma cells, using stable transfectants of P19 cells which expressed chicken lamin A. Once again, normal distribution of the introduced lamin was seen. They found that the transfected cells retained the antigenic and growth properties characteristic of undifferentiated P19; expression of lamin A did not induce differentiation in this system. Since lamins A and C expression in this system is a late event that follows expression of the differentiated phenotype (Mattia et al., 1992), it appears logical that unscheduled expression of these proteins would not initiate a cascade of differentiation events. The authors also found that the presence of lamin A did not inhibit retinoic acid-induced differentiation. Neither expression of markers of the mature phenotype, nor the time course of response to retinoic acid appeared to be affected in the transfected cells. This result was more surprising. If lamins A and C expression serves to limit the developmental plasticity of the cell, unscheduled expression of lamin A would be expected to interfere with or limit the response to inducers of differentiation. The fact that lamins A and C are usually coexpressed in P19 and most other cell types may be significant in terms of their role in differentiation. The modifications of the lamina structure and function brought about by incorporation of lamins A and C may not be reproduced accurately or irreversibly by lamin A alone. This possibility could be examined by transfection experiments that introduce both lamins. In addition, Peter and Nigg (1992) propose that lamin A/C knockout

experiments using an antisense approach could shed light on whether lamins A and C play a necessary role in differentiation.

Taken together, the results of the experiments performed for this thesis and literature reports of lamins A and C expression in a number of cell systems strongly suggest that induction of these proteins is a late event in differentiation, not coincident with expression of cytoplasmic IFP. Lamins A and C appear to be expressed some time after restriction of the developmental program to a given pathway and following expression of the differentiated phenotype. The expression of lamins A and C in HL-60 and other cell systems appears to be associated with the loss of developmental plasticity of response to changes in environment. Expression of these proteins was associated with loss of ability to change the physiological state in macrophages, for example. Recent transfection experiments have demonstrated that expression of lamin A alone does not restrict developmental plasticity (Peter and Nigg, 1992). Future work using a model that co-expresses lamins A and C or lamin A/C knockout experiments may shed further light on the contribution of these proteins to the differentiation process.

CONCLUSION

The inducible promyelocytic HL-60 cell line was exploited in order examine the context of lamins A and C protein expression during differentiation. HL-60 are lamins A/C-negative in the undifferentiated state, but are known to express these proteins in response to TPA, an inducer of differentiation towards macrophages or DMSO, an inducer of the granulocytic path (Paulin-Levasseur et al., 1989a). To address the question of whether lamins A/C expression was associated with the differentiated phenotype, a detailed kinetic analysis of these proteins in HL-60 cell populations treated with either TPA or Vitamin D_3 was made.

Induction of HL-60 cell differentiation by vitamin D_3 did not lead to expression of either lamins A/C or the cytoplasmic IFP vimentin. Treatment with TPA, on the other hand, resulted in expression of these proteins in the vast majority of cells within 24 hours.

Lamins A/C expression was clearly not associated with growth arrest of the vitamin D_3-treated cells. In TPA-induced populations, kinetic analysis revealed that lamins A and C expression followed inhibition of replication. These results confirmed and extended a previous study of lamins A/C expression in TPA-treated HL-60 (Paulin-Levasseur and Julien, 1992).

It was determined that lamins A and C expression was not coincident with expression of the differentiated phenotype in HL-60. Vitamin-treated populations demonstrated change

in marker expression and adherence of half of the cells to the substratum in the absence of lamins A/C. Kinetic analysis of TPA-treated populations demonstrated that lamins A/C expression followed dramatic morphological changes and adherence of the majority of the cells to the substratum, as well as vimentin expression. Expression of lamins A and C was not coupled to the expression of the differentiated phenotype in the HL-60 cell system, including expression of the cytoplasmic IFP vimentin.

These results suggested that lamins A and C expression is a very late or post differentiation event. This proved to be the case in the murine peritoneal macrophages studied as a physiologically normal control cell type. Confirming a previous report (Röber et al., 1990b), it was determined that these cells expressed lamins A and C only after a period of time spent in primary culture. In these cells, lamins A/C expression was not reflective of lineage commitment, but of physiological state. Literature reports provided other examples of post differentiation-expression of lamins A and C in epidermal and neural tissues of the mouse embryo (Röber et al., 1989) and in embryonic carcinoma cells (Mattia et al., 1992).

In summary, lamins A and C expression in both vitamin D_3- and TPA-treated HL-60 cells was a very late or post-differentiation event, which followed cessation of growth and expression of the differentiated phenotype. Confirming a previous report (Röber et al., 1990b), lamins A/C expression in macrophages was found to follow this pattern as well, being determined by the physiological state of the cells. These results are compatible with the hypothesis of the group led by Röber, that expression of lamins A and C protein is associated with loss of all developmental plasticity in the face of future environmental change.

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