INTRODUCTION

Cell cycle progression in mammalian cells is regulated by the family of cyclin-dependent serine-threonine protein kinases (cdks) (for reviews see Müller et al., 1993; Sherr, 1993; Hunter, 1993). Different cdk genes act at distinct phases of the cell cycle, the precise timing of their action being determined by their cell cycle-specific expression, their phase-specific phosphorylation and their interaction with specific cyclins. It is, for example, well established now that cdk2/cyclin E complexes exert their function in late G1 and S (Dulic’ et al., 1992; Koff et al., 1992, 1993; Ohtsubo and Roberts, 1993; Tsai et al., 1991), while cdc2/cyclin B complexes promote the progression from G2 into mitosis (reviewed by Pines and Hunter, 1990b). The association of cyclin A with cdk2 and cdc2, on the other hand, seems to be a prerequisite for the progression through S phase and G2 (Walker and Maller, 1991; Girard et al., 1991; Pagano et al., 1992; Tsai et al., 1993), whilst complexes of D-type cyclins and cdk4 are thought to play a role in G1→S transition (Matsushime et al., 1992; Quelle et al., 1993; Xiong et al., 1992a; Baldwin et al., 1993).

Some of the cell cycle regulatory proteins are regulated not only with respect to their activity, but also at the level of expression. Thus, the levels of cyclin A, cyclin B, cyclin E, cdc2 and cdc25 mRNA cycle periodically and show peak levels of expression that are consistent with their phase-specific functions (Pines and Hunter, 1989, 1990a; Sadhu et al., 1990; Lew et al., 1991; Dalton, 1992; Dulic’ et al., 1992; Koff et al., 1992). The regulation of other cell cycle genes in normally proliferating cells is however unclear. Thus D-type cyclins and cdk4 are induced by mitogens during the G0/S transition (Matsushime et al., 1991; Won et al., 1992; Sewing et al., 1993), but it is unclear whether their expression fluctuates during the cell cycle, either because this question has not been analysed or because of conflicting data (Baldin et al., 1993; Jansen-Dürr et al., 1993; Motokura et al., 1991; Sewing et al., 1993). In the present study, we have therefore addressed this question, using the human myeloid leukaemia cell line HL-60 (Koeffler and Golde, 1980). Fractions of HL-60 cells obtained by counter-flow elutriation were used to analyse the expression of five cyclin and two cdk genes in normally cycling, non-synchronised cell populations. Our results indicate that cyclin A, cyclin B and cdk2, and surprisingly an up-regulation of cyclin D1 in TPA-induced macrophage-like cells; (ii) a down-regulation of cyclin E in retinoic acid-induced granulocytic cells; and (iii) a decreased abundance of cyclin D1 and D2, but high levels of cyclin A, B and E RNA in DMSO-induced granulocytic cells. These observations suggest that the mechanisms leading to a differentiation-associated cell cycle arrest are lineage-specific, and that the sustained expression of cyclin and cdk genes does not interfere with the induction of terminal differentiation.

Key words: myeloid cell, differentiation, phorbol ester, retinoic acid, DMSO, cell cycle, gene regulation, cyclin, cdk gene
(Rovera et al., 1979), whilst retinoic acid (RA) or dimethyl sulfoxide (DMSO) induce granulocytic differentiation (Collins et al., 1978; Breitman et al., 1980). In addition, differentiation occurs relatively synchronously and affects basically the entire cell population. Treatment of HL-60 cells with either of the above inducers leads to an irreversible block in G1, raising the question of whether it may be the lack of expression of certain cell cycle genes that is responsible for this cell cycle arrest. In agreement with such a hypothesis, it has recently been reported that the expression of several G2-specific cell cycle genes, i.e. cdc2, cdc25, wee-1 and cyclin B are down-regulated in HL-60-derived macrophage-like cells (Hass et al., 1992; Horiguchi-Yamada et al., 1993). In addition, the induction of erythroid differentiation in vitro has been described to be accompanied by the decreased expression of cyclin A and an underphosphorylation of pRB (Kiyokawa et al., 1992, 1993). In contrast, no significant changes in the expression of cdc2, cdc25, wee-1 and cyclin B were seen in DMSO-induced granulocytic cells (Horiguchi-Yamada et al., 1993). To address this issue in further detail, we have performed a comprehensive analysis of cell cycle gene expression in differentiating HL-60 cells induced with TPA, DMSO or RA, and included in this study the G1-associated cell cycle genes cyclin D1, D2 and E as well as cdc2 and cdk4, whose expression in differentiating cells has not been analysed to date. Our observations lead to the conclusion that the induction of terminal differentiation in HL-60 cells affects specific sets of cell cycle genes, and that the observed expression patterns are characteristic of the respective lineage and chemical inducer used, even though all three treatments cause a cell cycle block in G1. In this context, the probably most surprising results are the high levels of cyclins A, B and E and the up-regulation of cyclin D1 in terminally differentiated G1-arrested cells of specific lineages. Our observations suggest that different, lineage-specific mechanisms may play a role in the induction of differentiation-associated cell cycle arrest.

MATERIALS AND METHODS

Growth and differentiation of HL-60 cells
HL-60 cells were grown in RPMI-1640 medium supplemented with 10% FCS. Differentiation to macrophage-like cells (Rovera et al., 1979) was induced by exposure of the cell to 10^−6 M TPA for 30 minutes and subsequent incubation in normal growth medium for 2 days. Induction of granulocytic differentiation (Collins et al., 1978; Breitman et al., 1980) was accomplished by exposing the cells to 10^−6 M RA or 1.25% DMSO for 8 days.

FACS analysis
FACS analysis of Hoechst 33258 stained HL-60 cells and counter-flow elutriation have been described previously (Wick et al., 1994).

RNA isolation and analysis by reverse transcriptase-PCR
RNA was isolated according to the method of Chomczynski and Sacchi (1987). Reverse transcription of RNA into cDNA and quantitative PCR analysis were performed as described (Sewing et al., 1993). Quantification was performed by β-radiation scanning of the gels using a Molecular Dynamics PhosphorImager. Data were corrected using the L7, GAPDH or β-actin signal as the standard. The following primer pairs were used for PCR amplification:

<table>
<thead>
<tr>
<th>Primer Pair</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin A (Kang et al., 1990)</td>
<td>(5´-primer): 5´-GTCAAGGGCTCCATATGCTGGCCACTCTCCG-3´ (106-125)</td>
</tr>
<tr>
<td>Cyclin B (Pines and Hunter, 1989)</td>
<td>(5´-primer): 5´-CCATATGTGGCCACACTCTCCG-3´ (817-840)</td>
</tr>
<tr>
<td>Cyclin D1 (Kong et al., 1991)</td>
<td>(5´-primer): 5´-GGTGGGCTCCATATGCTGGCCACTCTCCG-3´ (1378-1401)</td>
</tr>
<tr>
<td>Cyclin D2 (Kong et al., 1992b)</td>
<td>(5´-primer): 5´-GGTGGGCTCCATATGCTGGCCACTCTCCG-3´ (202-222)</td>
</tr>
<tr>
<td>Cyclin E (Law et al., 1995)</td>
<td>(5´-primer): 5´-ATCGAATTAATCTAAGCCTCCTGCAAC-3´ (171-190)</td>
</tr>
<tr>
<td>Cdk2 (Tsai et al., 1991)</td>
<td>(5´-primer): 5´-GAGGATGGCTTGAGAAATCTCCGAGAAG-3´ (170-189)</td>
</tr>
<tr>
<td>Cdk4 (Hanks, 1987)</td>
<td>(5´-primer): 5´-ACCCCGGCGGCTTGAGAAATCTCCGAGAAG-3´ (278-298)</td>
</tr>
</tbody>
</table>

RESULTS

Cell cycle phase-specific expression of cyclin and cdk genes in HL-60 cells
The expression of cyclin and cdk genes during the cell cycle has been analysed in a number of studies (see Introduction). In the majority of these analyses, synchronised cells that were used that had been released from a metabolic block or a state of quiescence by mitogen stimulation. It is clear that such experimental strategies might lead to results that do not reflect the patterns of gene expression occurring during a normal cell cycle. Thus, we have observed inappropriate gene expression and aberrant transcripts in human fibroblasts synchronised at the G1/S boundary by a thymidine/aphidicolin block, e.g. in the case of γ-actin and cdc25C genes (our unpublished observations). The reported discrepancies with respect to cyclin D1 expression during the cell cycle (see Introduction) may also be, at least in part, attributable to synchronisation artefacts. We therefore sought to perform a systematic study of cell cycle gene expression in non-synchronised, normally cycling HL-60 cells. Owing to their homogeneity in cell size, HL-60 cells are ideally suitable for separation by counter-flow elutriation. This technique was therefore chosen to analyse the expression of seven different cyclin and cdk genes during HL-60 cell cycle progression. A typical HL-60 elutriation profile is shown in Fig. 1a. Of the nine fractions collected, the first three contained >95% G1 cells. A progressive decline in G1 cells and a continu-
The continuous increase in the number of S and G2 cells was seen in subsequent fractions; the last two fractions, F8 and F9, containing 70-80% cells in G2/M, and only 10% in G1. The elutriation procedure thus yielded a ~10-fold enrichment of G1 cells in fraction F1 and a ~40-fold enrichment of G2 cells in F9.

RNA analyses were performed by reverse transcriptase PCR (RT-PCR), since especially with elutriated cells only limited amounts of RNA were available. The RT-PCR established in our laboratory (Mumberg et al., 1991) yields results that are basically identical to the data obtained by Northern blotting, as previously shown by a direct comparison (Lucibello et al., 1993). The experimental strategy included the following precautions: (i) the number of PCR cycles was kept low in order to obtain an exponential amplification of the PCR products, which was possible by the incorporation of radioactive precursor nucleotides and evaluation by autoradiography and β-radiation scanning; (ii) all results were standardized using the signal obtained with L7 or GAPDH, whose expression is independent of cell proliferation; (iii) all experiments were performed with at least two independent cDNA preparations; (iv) only fluctuations of at least 2-fold were considered significant; and (v) to control for DNA contamination, primers were designed that spanned at least one exon-intron boundary (if information on the gene structure was available).

Three genes showed a clear cell cycle phase-dependent expression (Fig. 1b) that was in agreement with results previously obtained with other cell lines and experimental approaches (Pines and Hunter, 1989, 1990a; Lew et al., 1991; Dulic’ et al., 1992; Koff et al., 1992). These genes were cyclins A, B and E, as well as the recently identified alternative splice form of cyclin E, cyclin Es (Sewing et al., 1994). The earliest expression was seen in the case of the two cyclin E forms. Both cyclin E RNAs showed the lowest abundance in fractions F1 and F9, a strong increase was seen in fraction F2 and a similarly clear decline was seen in fraction F9 relative to F8. These results indicate that cyclin E and Es mRNAs increase at later stages of G1, reach peak levels in S, and decrease again in G2. Cyclin E expression was followed by the induction of cyclin A during S phase and G2. The increase in cyclin A RNA levels was followed by the induction of cyclin B, which reached maximum expression in G2 shortly after cyclin A. In contrast to these three cyclin genes, two others, cyclin D1 and D2, did not show any significant regulation during the HL-60

**Fig. 1.** Expression of cell cycle genes in normally cycling HL-60 cells separated by counter-flow elutriation. (a) FACS analysis of cell cycle distribution in 9 different fractions obtained by elutriation (F1 to F9). (b) Analysis of mRNA expression by RT-PCR and quantification of the results by β-radiation scanning.
a peak of expression was reproducibly seen in fractions F4 to F6, i.e. in cells at G1/S and/or in S phase.

Cell cycle kinetics and expression of marker genes in differentiated HL-60 cells

Before turning to the question of cyclin and cdk gene expression in differentiating HL-60 cells, we determined the cell cycle kinetics and the expression of differentiation marker genes in cells induced with different agents along different differentiation pathways. The results of the cell cycle analysis (Fig. 2a) show that in untreated control cells approximately half of the population was in either S phase or G2. By contrast, in differentiated cell populations induced with either TPA, RA or DMSO only approximately 10% of the cells showed a DNA content characteristic of cells in S or G2. These observations clearly suggest that all three treatments lead to a block in the G1 phase of the cell cycle in at least 90% of the cell population. This result is in agreement with the appearance of differentiated macrophage-like or granulocytic cells detected by staining with Giemsa reagent (not shown).

We also analysed the expression of two genes that are considered as marker genes for myeloid differentiation, the proto-oncogene c-fms (Sariban et al., 1985) and the serine protease myeloblastin gene (Bories et al., 1989). The c-fms gene encodes the CSF-1 receptor (Sherr et al., 1985) and has been reported to be expressed in macrophages but not in granulocytes obtained after DMSO treatment of HL-60 cells (Sariban et al., 1985). The myeloblastin gene, on the other hand, has been described to be down-regulated in cells differentiating along either the monocytic or granulocytic lineage (Bories et al., 1989). Fig. 2b and c and Table 1 show that the expected patterns of expression were seen in most cases. Thus, the expression of myeloblastin dropped >30-fold in terminally differentiated cells obtained after exposure to either TPA, RA or DMSO. Also as expected, the expression of c-fms was induced >30-fold in the macrophage-like cells obtained after TPA treatment, whilst no significant change was seen in DMSO-treated cells. However, in contrast to the anticipated result exposure of HL-60 cells to retinoic acid resulted in a more than 50-fold induction of c-fms in the differentiated granulocytic cells. This finding clearly suggests that the granulocytic cells generated by RA or DMSO treatment are not identical. For this reason, all three inducers were used for analysing the expression of cell cycle genes in differentiating HL-60 cells.

Expression of cell cycle genes in differentiating HL-60 cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>TPA†</th>
<th>RA‡</th>
<th>DMSO‡</th>
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<tbody>
<tr>
<td>Cyclin A</td>
<td>↓&gt;40</td>
<td>⇔</td>
<td>⇔</td>
</tr>
<tr>
<td>Cyclin B</td>
<td>↓24</td>
<td>↓4</td>
<td>⇔</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>1.5</td>
<td>⇔</td>
<td>↓5</td>
</tr>
<tr>
<td>Cyclin D2</td>
<td>⇔</td>
<td>⇔</td>
<td>4</td>
</tr>
<tr>
<td>Cyclin E</td>
<td>↓4</td>
<td>⇔</td>
<td>10</td>
</tr>
<tr>
<td>Cyclin Es</td>
<td>↓4</td>
<td>10</td>
<td>⇔</td>
</tr>
<tr>
<td>cdk2</td>
<td>↓8</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>cdk4</td>
<td></td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>c-fms</td>
<td>↑&gt;30</td>
<td>↑&gt;50</td>
<td>⇔</td>
</tr>
<tr>
<td>Myeloblastin</td>
<td>↓&gt;50</td>
<td></td>
<td>▼&gt;30</td>
</tr>
</tbody>
</table>

*Numbers indicate factor of down-regulation (↓) or up-regulation (↑) in the terminally differentiated cells compared to normally cycling cells; ⇔, decrease in expression ≤2. The most pronounced changes seen with each of the 3 inducers are marked by shading.

†At 48 hours after a 30-minute exposure to TPA.
‡After 8 days of exposure to RA or DMSO.

Fig. 2. (a) FACS analysis of cell cycle distribution in differentiated HL-60 cells after exposure to TPA (30 minutes followed by 48 hours of incubation in normal medium), RA (8 days) or DMSO (8 days). (b,c) Expression of the differentiation marker genes c-fms and myeloblastin in HL-60 cells at different times after exposure to RA, DMSO or TPA. The analysis was carried out by RT-PCR as in Fig. 1b.
Regulation of cell cycle genes

HL-60 cells at different times after exposure to TPA, RA or DMSO were analysed for expression of cyclin A, B, D1, D2, E and Es, as well as cdk2 and cdk4 mRNA. The results of this study are shown in Figs 3 and 4 and are summarised in Table 1. TPA treatment led to a dramatic decrease in the abundance of cyclin A, cyclin B and cdk2 mRNA levels, which declined >40-fold, 24-fold and 8-fold, respectively (Figs 3, 4; Table 1).

A comparatively moderate decrease was observed for the two cyclin E RNA isoforms and cdk4, which showed a 3- to 4-fold lower expression in the terminally differentiated cells. While no significant alterations were seen with cyclin D2 RNA levels, the expression of cyclin D1 showed a 5-fold increase 1-2 days after TPA treatment. A completely different picture was obtained with RA-treated cells (Figs 3, 4; Table 1). In this case, only cyclin E and Es mRNA levels showed a strong down-regulation of approximately 10-fold in the terminally differentiated cells. Cyclin B and cdk2 showed only a 3- to 4-fold decrease while no significant down-regulation was seen with cyclins A, D1 and D2 as well as cdk4. DMSO-induced granulocytic cells showed a still different picture of cell cycle gene expression (Figs 3, 4; Table 1). In this case, the most pronounced decrease was seen with cyclins D1 and D2. All other cyclins did not show any significant down-regulation of RNA expression, while the abundance of cdk2 and cdk4 mRNA decreased approximately 3-fold. These results clearly indicate that cell cycle genes are regulated during the differentiation of HL-60 cells in a lineage-specific and inducer-dependent fashion (see Table 1).

DISCUSSION

The mammalian cell cycle is controlled by a specific set of cdc2-related serine-threonine protein kinases whose catalytic and regulatory subunits are members of the cdk and cyclin families, respectively (for reviews see Müller et al., 1993; Sherr, 1993; Hunter, 1993). A number of studies have shown that both cdks and cyclins are regulated at different levels, including expression, phosphorylation and protein interactions. For some of these genes, such as cyclin A, cyclin B and cdc2, there is very good evidence that their expression is cell cycle-phase-dependent, while the situation is unclear with other cell cycle genes, like for instance the D-type cyclins and cdk4 (see Introduction). In the first part of this study, we have therefore systematically analysed the expression of seven cyclin and cdk genes in normally proliferating HL-60 cells separated by counter-flow elutriation. A clear cell cycle-dependent regulation was seen with cyclins D1 and D2. All other cdks did not show any significant down-regulation of RNA expression, while the abundance of cdk2 and cdk4 mRNA decreased approximately 3-fold. These results clearly indicate that cell cycle genes are regulated during the differentiation of HL-60 cells in a lineage-specific and inducer-dependent fashion (see Table 1).

![Fig. 3. Autoradiograph of a RT-PCR experiment showing expression of cyclin A, B and D1 RNA in differentiating HL-60 cells after exposure to TPA, DMSO or RA. Exposure to TPA was for 30 minutes followed by incubation in normal medium.](image-url)
Fig. 4. Expression of cyclin and cdk genes in differentiating HL-60 cells after exposure to RA, DMSO or TPA. The analysis was carried out by RT-PCR as for Fig. 1b.
BrdU-labelling technique with human fibroblasts, although their immunoblot data are difficult to reconcile with this conclusion. Finally, Sewing et al. (1993) analysed the expression in the human fibroblast line WI-38 released from a thymidine/aphidicolin block and found no significant fluctuation in the expression of cyclin D1 mRNA. In agreement with this result, we have recently analysed cyclin D1 protein levels in normally cycling NIH3T3 cells by immunofluorescence, digital image analysis and cell sorting, and were unable to detect any significant fluctuations during the cell cycle (our unpublished observations). It is possible that some of these discrepancies are related to the specific synchronisation procedures used and perhaps due to cell line-specific differences. We have recently initiated a study addressing this problem in detail.

In the second part of this study, we have analysed the regulation of cyclin and cdk gene expression in differentiating HL-60 cells. Induction of macrophage-like differentiation by TPA, or granulocytic differentiation by either retinoic acid or DMSO, arrested cells in G1 and resulted in lineage-specific patterns of cell cycle gene expression (see summary in Table 1). Thus, the levels of cyclin A and B mRNA were dramatically reduced in TPA-induced cells, but surprisingly not in cells differentiated into granulocytic cells, as previously reported by others for cdc2, cdc25, wee-1 and cyclin B (Horiguchi-Yamada et al., 1993). A down-regulation of cell cycle genes whose products function in G2 has also been described for differentiating Friend cells (Kiyokawa et al., 1992, 1993), for the myeloid leukaemia cell lines U-937 and THP-1 after monocyctic differentiation (Hass et al., 1992) and for differentiating neuronal cells (Hayes et al., 1991). It thus seems, that the lack of down-regulation of G2-associated cell cycle genes is a specific phenomenon of the granulocytic pathways induced by RA or DMSO in HL-60 cells. Lineage-specific down-regulation was also observed in the case of the cyclin E gene (see Table 1). The abundance of both cyclin E and cyclin Es mRNA was greatly diminished in RA-induced cells, to a considerably lesser extent in TPA-treated cells, and hardly at all in the granulocytic cells obtained after exposure to DMSO. Similarly, a strong decrease in the level of cdk2 RNA was seen specifically in TPA-induced cells. Probably the most surprising result was the increased expression of cyclin D1, a proliferation-promoting gene (Baldin et al., 1993), exclusively in HL-60 cells differentiated by TPA, although the same gene was down-regulated in DMSO-induced granulocytic cells.

Our results indicate that only specific cyclin genes are down-regulated, and even G2-specific cyclin mRNAs, like cyclin A and B, may be expressed at high levels in the G1-arrested, terminally differentiated cells. Likewise, G1 cyclins such as cyclin D1 may be down-regulated during the differentiation along one lineage, but up-regulated during the differentiation along another. These observations lead to the conclusion that the regulation of cell cycle genes in the process of terminal differentiation is lineage-specific and inducer-dependent. Our observations also suggest that the down-regulation of cyclin A, B, D and E genes is not a prerequisite for the induction of terminal differentiation and the concomitant cell cycle block in G1. The diminished expression of one or more cyclin mRNAs in each of the lineages tested in this study may however suggest that the down-regulation of cyclin genes does indeed play a role in terminal differentiation, but that the mechanisms are pathway-specific. Future studies analysing the effect of ectopically expressed cyclins in HL-60 cells may help to provide an answer to this question.

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