Cell Cycle Dependent Regulation of cdc2 mRNA in Mouse Fibroblasts: Requirement of Protein Synthesis and of Continued Mitogenic Stimulation

CHRISTINE BUQUET-FAGOT,* DOMINIQUE FAGOT, AND JAN MESTER
INSERM U.55, 184 rue du Fbg St Antoine, 75771 Paris cedex 12, France

In the chemically transformed mouse fibroblasts (BP-A31) placed in a serum-free medium, the cdc2 mRNA content decreases in parallel with the cessation of [3H]thymidine incorporation. Extinction of the cdc2 gene expression is also observed in BP-A31 cells overexpressing the human c-myc oncogene. At quiescence, the cdc2 gene expression can be reinduced with serum or with other mitogens such as insulin or 12-O-tetradecanoyl phorbol 13-acetate (TPA). The kinetics of induction is characterized by a lag period which differs according to the mitogen used and reflects the length of the G1 phase (4-6 h with insulin or serum, 9-12 h with TPA). The cdc2 mRNA accumulation is prevented when protein synthesis is blocked with cycloheximide, also if the drug is added at a time when the synthesis of cdc2 mRNA is already under way. Similarly, removal of the mitogen leads to a cessation of the cdc2 mRNA accumulation. These results suggest that the increased expression of the cdc2 gene is mediated by (a) short-lived, growth factor-regulated protein(s).

The p34 cdc2 is a serine/threonine protein kinase originally identified as the product of the cdc2 gene of Schizosaccharomyces pombe and of the cdc28 gene of Saccharomyces cerevisiae (Nurse and Bisset, 1981; Nasmyth and Reed, 1980). In the yeast, this protein kinase activity is necessary for both G1/S transition and for the initiation of mitosis (Nurse and Bisset, 1981; Piggott et al., 1982). In higher eukaryotes, p34 cdc2 complexes with cyclin B to form the growth-associated kinase I which regulates the initiation of mitosis as well as meiosis in the oocyte (Dunphy et al., 1988; Arion et al., 1988; Labbé et al., 1989). Recent studies indicate that the p34 cdc2 (or a related kinase) may be necessary for the replication of chromosomal DNA in the cells of higher eukaryotes, although its precise role remains unclear (D'Urso et al., 1990; Blow and Nurse, 1990; Fotedar and Roberts, 1992; Dutta and Stillman, 1992). The enzymatic activity of the p34 cdc2 protein is regulated by its state of phosphorylation which fluctuates with the stage of the cell cycle (review Moreno et al., 1989); dephosphorylation on tyrosine is required for its activity at the mitotic metaphase (Gould and Nurse, 1989; Morla et al., 1989; Norbury et al., 1991). In yeast cells, the amount of p34 cdc2 protein and mRNA do not vary during the cell division cycle (Durkacz et al., 1986); in contrast, in mammalian cells the cdc2 mRNA level has been shown to differ between quiescence and proliferative state (Lee et al., 1988; Shaun and Thomas, 1989; Furukawa et al., 1990). Moreover, the cdc2 gene is expressed and transcriptionally regulated as a function of the cell cycle (McGowan et al., 1990; Surnacz et al., 1992; Welch and Wang, 1992; Dalton, 1992).

Received August 19, 1992; accepted November 2, 1992.
*To whom reprint requests/correspondence should be addressed.
cells were allowed to enter quiescence during the next 2–3 days. In a parallel set of dishes, \[^{3}H\]thymidine (Amersham, Les Ulis, France) incorporation (2 μCi/ml) was evaluated during 2 h pulses at the time points as shown (Gray et al., 1987).

**BP-A31 derived cell lines expressing human c-myc gene**

BP-A31 cells were transfected by the calcium phosphate procedure with the c-myc expression vector (obtained from R. Offringa) containing the XbaI-EcoRI fragment of the human c-myc (exons 2 and 3) under the control of the SV40 early promoter, dihydrofolate reductase and hygromycin coding sequences both under the control of the HSV thymidine kinase promoter. Hygromycin resistant clones were isolated, amplified, and referred to as BP-Myc cell lines. Cell culture conditions were as for BP-A31 cells.

**Analysis of RNA**

Cells in P100 dishes were chilled on ice, washed, and total RNA was isolated using the urea/lithium chloride precipitation procedure (Auffray and Rougeon, 1980). Poly (A') RNA were selected by chromatography on oligo (dT) cellulose. Twenty micrograms of total or 5 μg of poly (A') RNA were size fractionated on agarose (1%)-formaldehyde (2.2 M) gel, transferred onto nylon membrane (Hybond N; Amersham), fixed by U.V. exposition, and hybridized at 42°C with \[^{32}P\]-labeled probes in a solution containing 50% formamide, 0.45 M NaCl, 3 mM ethylene diamine tetraacetic acid (EDTA), 0.1% sodium dodecyl sulfate (SDS), 75 mM phosphate buffer, pH 7.4. The filters were washed at 45°C in 0.15 M NaCl, 15 mM sodium citrate, and exposed with XAR 5 Kodak film and intensifying screen. The quantification was carried out by densitometry scanning (Biocom, Paris, France). The results shown were verified by repeated experiments using RNA from independent cell culture.

**Probes**

The probes were rat α-tubulin cDNA (Lemishka et al., 1981), human elongation factor (hEF; Brands et al., 1986) used as internal standards; cdc2 mouse cDNA (kindly provided by P. Nurse); human c-myc 3rd exon (EcoRI-ClaI fragment; Dalla Favera et al., 1982). The probes were \[^{32}P\] labeled by the Multiprime DNA labeling system (Amersham).

**Growth factors and chemicals**

Bovine insulin was obtained from Boehringer, \[^{3}H\]thymidine and \[^{32}P\]dCTP were purchased from Amersham, TPA, 3-isobutyl-1-methyl xanthine (IBMX), and dexamethasone were from Sigma (St. Louis, MO, USA). Other compounds (reagent grade) were from usual commercial sources.

**RESULTS**

**cdc2 mRNA content during serum deprivation and mitogenic restimulation**

A strong cdc2 mRNA signal was detected in exponentially growing cells. Serum starvation caused a progressive decrease in the cdc2 mRNA content (half-life of approximately 18 h) in correlation with the arrest of \[^{3}H\]thymidine incorporation (Fig. 1). After 3 days of starvation, the cdc2 mRNA level was about 8% of that found in exponentially growing cells.

Quiescent BP-A31 cells accomplish the full cell division cycle when stimulated either with FCS or with individual mitogens. Maximum of thymidine incorporation (evaluated by the rate of \[^{3}H\]thymidine incorporation) was observed after 14–17 h of incubation with FCS or insulin, and 20–23 h when TPA was used as mitogen (data not shown; cf. Buchou et al., 1989).

Following mitogenic stimulation, an increase in the cdc2 mRNA content could be detected after a delay of several hours; the delay was shorter with insulin and with FCS (4–6 h) than with TPA (9–12 h) (Fig. 2). These results show that the accumulation of cdc2 mRNA is not restricted to a specific mitogenic pathway.

The cdc2 mRNA is relatively stable in cells synchronously induced to resume the cell division cycle: 10 h after serum stimulation, the decrease in the cdc2 mRNA content observed in the presence of actinomycin D (10 μg/ml) corresponded to a \(t_{1/2}\) of approximately 10 h (data not shown).

**Induction of cdc2 mRNA gene expression is a protein synthesis-dependent process**

The delay observed before cdc2 mRNA accumulation suggested that cdc2 expression depends on early G1
phase events, such as the expression of the G0/G1 transition genes. In order to test the involvement of protein synthesis in the induction of cdc2 gene expression, re-stimulation of quiescent cells was carried out in the presence of cycloheximide (Fig. 3). Under these conditions, the accumulation of cdc2 mRNA was totally abolished. Even when added as late as 5 h after the mitogen, cycloheximide significantly inhibited cdc2 gene expression, suggesting that the expression of cdc2 gene requires the synthesis of short-lived protein(s). The possible involvement of the c-myc protein in the cdc2 gene expression has been suggested by experiments based on the use of antisense c-myc oligonucleotides (Furukawa et al., 1990). However, we have also observed the elimination of cdc2 mRNA during incubation in a serum-free...
medium in BP-A31 cells stably transfected with a construct expressing the human c-myc gene placed downstream of the SV40 early promoter (Fig. 4). This result confirms that the c-myc gene product is not sufficient to ensure cdc2 gene expression which necessitates further action of "progression" factors such as insulin/IGF1 (Surmacz et al., 1992) or TPA (see above).

### Accumulation of cdc2 mRNA requires continuous mitogenic stimulation

The necessity of a continuous presence of growth factors for cdc2 gene expression was tested in experiments where quiescent cells were restimulated with insulin during 6 h or 9 h and then maintained in the absence of the growth factor for 3–6 h. Insulin was preferred in these experiments as TPA cannot be removed from the culture medium, and serum also contains growth factors which adsorb to the dish and continue to exert action of "progression" factors such as insulin IGF1 (Surmacz et al., 1992) or TPA (see above).

<table>
<thead>
<tr>
<th>TIME (h)</th>
<th>TPA</th>
<th>INS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>IBMX</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>hEF</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>cdc2</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Inhibitors of G1 progression act selectively on cdc2 gene expression

The different mitogenic signalling pathways in BP-A31 cells can be distinguished by their differential sensitivity to inhibitors acting in G1 phase, such as drugs inducing intracellular cAMP accumulation (IBMX; an inhibitor of cyclic nucleotide phosphodiesterase) or glucocorticoids (dexamethasone; a synthetic glucocorticoid). While the mitogenic effects of TPA are completely abolished by IBMX, the mitogenic response dependent on the activation of IGF1 receptors is only retarded but not prevented by this drug (Buchou et al., 1988). Dexamethasone (2 × 10^{-7} M) also inhibits mitogenic effects elicited by phorbol esters but does not affect mitogenic activity of insulin (Fagot et al., 1991). In correlation with their signalling pathway-specific effects, both IBMX and dexamethasone blocked the accumulation of cdc2 mRNA in cells stimulated with TPA but not with insulin (Fig. 6, 7).

### DISCUSSION

The G1 phase may be interpreted as a cascade of growth factors-induced events leading to the accumulation and the activation of molecules (proteins) required to trigger DNA replication as well as for subsequent processes. The growth factor-induced gene expression evolves in the course of progression: certain genes (c-fos, c-myc, c-jun) are expressed in early G1 phase whereas others, including cdc2, late in the G1 phase. In quiescent BP-A31 cells, the cdc2 gene expression is low and can be induced by agents which act as mitogens in these cells. The induction is not restricted to a specific mitogenic pathway since insulin (binding to IGF1 receptors and activating a tyrosine kinase pathway) and TPA, a protein kinase C activator, both cause an increase in the cellular content of cdc2 mRNA. The kinetics of the cdc2 mRNA accumulation is characterized by a lag time which differs according to the mitogen (4–6 h with insulin and serum, 9–12 h with TPA) and reflects the duration of the G1 phase.

Our experiments indicate that the accumulation of cdc2 mRNA begins in late G1 phase. Strong increase in the cell content of cdc2 mRNA is observed as early as 6 h after restimulation of quiescent cells with FCS or insulin, i.e., well before the increase of [\(^{3}H\)]thymidine incorporation.

The induction of cdc2 mRNA accumulation is an indirect consequence of the mitogen-initiated signal transduction. This is illustrated by the fact that drugs which selectively inhibit the TPA-induced G1 phase progression (such as dexamethasone or intracellular cAMP) also prevent the increase in the cdc2 content in TPA-stimulated cells but not in cells stimulated by insulin. The effects of TPA on "immediate early" gene expression are not affected; for instance, the induction of c-fos by TPA takes place also in cells cultured with dexamethasone (Fagot et al., 1991). Unlike the G0/G1 transition genes (Greenberg and Ziff, 1984; Ryseck et al., 1988), the induction of the cdc2 gene expression requires continued protein synthesis and is totally prevented by cycloheximide. The expression of other late
G1 genes (thymidine kinase and PCNA/cyclin) has also been shown to require protein synthesis in early G1 phase (Jaskulski et al., 1988). If cycloheximide is added at the time when cdc2 accumulation is already under way (5 h after restimulation of quiescent cells with insulin), further increase in the cdc2 mRNA content is blocked. These observations suggest the existence of (a) short-lived intermediary protein(s), growth factor regulated, which are necessary for the expression of the cdc2 gene. Experiments where insulin was removed from the culture medium after 5 or 3 h after the hypoth-
thesis: the accumulation of cdc2 mRNA ceased within 3-6 h of the absence of the growth factor. The effect of the removal of the growth factor is slower than that of the addition of cycloheximide, possible as a consequence of the fact that the ligand-receptor interaction has trig-
gered processes which can continue for some time even
in the absence of the ligand in the external medium, whereas the effect of blocking protein synthesis will be immediate. The identity of such growth factor-regu-
lated intermediary proteins needed for the expression of cdc2 gene is not known at present. At least two possi-
bilities may be considered: 1) c-myc protein has been
implicated in the induction of cdc2 mRNA; however, in
the BP-A31 cells, the expression of the c-myc gene is deregulated (Campisi et al., 1984), and we have con-
firmed its constitutive expression at quiescence (Bu-
chou et al., 1989). The additional constitutive expres-
sion of exogenous c-myc in stably transfected BP-A31 cells also did not prevent the elimination of cdc2 mRNA during culture in a serum-free medium. While myc protein appears to be necessary for the cell cycle pro-
gression and the increase in cdc2 mRNA level (Dalton,

1992), clearly it is not sufficient in the absence of con-
tinuous growth factor stimulation. 2) The short-lived, growth factor-regulated protein could be a factor neces-
sary for the initiation of transcription of the cdc2 gene,
such as E2F (Dalton, 1992) or a transcription interme-
tary factor required for E2F-dependent transcription
activation.

ACKNOWLEDGMENTS

This work was supported in part by grant no. 6921 of
the Association pour la Recherche sur le Cancer (Villejuif, France). C.B.-F. was supported by a doctoral
grant from Ministère de la Recherche et de la Technol-
ogie. We thank Dr. G. Rosselin for his interest and
encouragement, and Y. Issoulié for the photographs.

LITERATURE CITED

Arion, D., Meijer, L., Brizuela, L., and Beach, D. (1988) cdc2 is a
component of the M phase-specific histone H1 kinase: Evidence for
Auffray, C., and Rougeon, F. (1980) Purification of mouse immuno-
globulin heavy-chain messenger RNAs from total myeloma RNA. Eur.
J. Biochem., 103:303-314.
Blow, J., and Nurse, P. (1990) A cdc2-like protein is involved in the
Buchou, T., and Mester, J. (1990) The fibroblasts growth factor-depen-
dent mitogenic signal transduction pathway in chemically trans-
formed mouse fibroblasts is similar but distinct from that initiated by phorbol esters. J. Cell. Physiol. 149:559-565.
Buchou, T., Charollais, R.H., and Mester, J. (1988) Involvement of
serum factors absorbed to the dish in response of cycloheximide-
Buchou, T., Charollais, R.H., Fagot, D., and Mester, J. (1989) Mitogen-
activity of phorbol esters and insulin-like growth factor 1 in chemically transformed mouse BP-A31: Independent effects and dif-
ferential sensitivity to inhibition by 5-isobutyl-1-methyl xanthine.
Campisi, J., Gray, H.E., Pardee, A.B., Dean, M., and Sommesein,
Charollais, R.H., Buchou, C., and Mester, J. (1989) Butyrate blocks the
accumulation of CDC2 mRNA in late G1 phase but inhibits both the
Dallapiccola, B., Gelmania, E.P., Martinotti, S., Franchini, G., Papas,
T.S., Gallo, R., and Wong-Staal, F. (1982) Cloning and characteri-
dation of different human sequences related to the onc gene (c-myc) of
avian myelocytomatosis virus (MC28). Proc. Natl. Acad. Sci. USA,
79:698-701.
J., 11:1767-1804.
xenopus cdc2 protein is a component of MPF, a cytoplasmic regula-
cell cycle control gene of the fission yeast Schizosaccharomyces pombe.
EMBO J., 5:369-379.
Cell cycle control of DNA replication by a homolog from human cells of
the p34 cdc2 protein kinase. Science, 250:786-791.
a human cell DNA replication factor, ROA, and activate DNA replica-
dation. EMBO J., 11:2189-2199.
Fagot, D., Buquet-Fagot, C., and Mester, J. (1991) Antimitogenic ef-
fects of dexamethasone in chemically transformed mouse fibro-
ylation of RAP32 occurs within the replication initiation complex.
EMBO J., 11:2177-2187.
Furukawa, Y., Pinwlick-Worms, H., Ernst, T.J., Kanakura, Y., and
Griffin, J.D. (1990) cdc2 gene expression at the G1 to S phase transition
transition in chemically transformed 3T3 cells. Exp. Cell Res.,
169:95-104.
Jaskulski, D., Gatti, C., Travali, S., Calabretta, B., and Baserga, R.
(1988) Regulation of the proliferating cell nuclear antigen cyclin and
thymidine kinase mRNA levels by growth factors. J. Biol. Chem.,
263:10175-10181.
Labbé, J.C., Capony, J.P., Caput, D., Cavardore, J.C., Derancourt,
starfish oocytes at first meiotic metaphase is a heterodimer con-
taining one molecule of cdc2 and one molecule of cyclin B. EMBO J.,
8:3053-3058.
expression and phosphorylation of a possible mammalian cell-cycle
Nucleotide sequence and evaluation of a mammalian α-tubulin messen-
of the human M-phase promoting factor catalytic subunit p34 dur-
Moreno, S., Hayles, J., and Nurse, P. (1989) Regulation of the cell cycle
Nasmyth, K.A., and Reed, S.I. (1980) Isolation of genes by complemen-
Acad. Sci. USA, 77:2119-2123.
Norbury, C., Blow, J., and Nurse, P. (1991) Regulatory phosphoryla-
tion of p34cdc2 protein kinase in vertebrates. EMBO J., 10:3321-
3329.


