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THE EFFECT OF CYCLOHEXIMIDE ON THE EXPRESSION OF CELL CYCLE DEPENDENT GENES*

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We have investigated the inducibility of several cell cycle-dependent genes (plus control sequences, not expressed in a cell cycle-dependent manner) in the presence of cycloheximide, an inhibitor of protein synthesis. The genes studied include: 1) five cDNA clones that are preferentially expressed in the G_1 phase of the cell cycle: KC-1, JE-3, 2F1, 4F1 and 2A9; 2) one gene preferentially expressed in late G_1/S phase: histone H3; and 3) the cell cycle-dependent oncogene p53. All the genes studied are induced by serum even in the presence of cycloheximide. Previous results in the literature have shown that 2 other oncogenes, c-myc and c-fos, can be induced by growth factors in the presence of cycloheximide. Together with our results, these findings indicate that protein synthesis is not required for the induction of at least nine cell cycle genes by growth factors.

Cell cycle-dependent genes are operationally defined as genes that are preferentially expressed in a specific phase of the cell cycle (1). There are a number of well-characterized animal genes whose expression is cell cycle-dependent. These include histone genes (2-4), c-myc (5,6), c-ras (6-8), c-fos (9-12), p53 (13), thymidine kinase (14), calmodulin (15), actin (6) and dihydrofolate reductase (16,17 but see some reservations in 14 and 18). Other cell cycle-dependent genes have been isolated as cDNA clones (19-24) by differential screening of cDNA libraries.

The fact that certain genes are expressed in a cell cycle-dependent manner does not, of course, mean that they regulate cell cycle progression. However, the fact that some oncogenes are expressed in a cell cycle-dependent manner

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Abbreviation: CX, Cycloheximide

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(5-13) suggests that at least some genes so expressed may play a role in cell cycle progression and, indeed, such a role has been proposed for c-myc, c-fos, c-ras and p53. Both c-myc and c-fos are inducible by growth factors in the presence of concentrations of cycloheximide (CX) that completely suppress protein synthesis (5,12). In fact, c-myc can be induced by CX in the absence of mitogenic stimuli (25). Similar findings have been reported for two cDNA clones that are induced in quiescent Balb/c-3T3 cells by platelet-derived growth factor (21). These findings suggest that at least 4 cell cycle genes whose expression is rapidly increased by growth factors are primary responders, i.e., do not require protein synthesis. In this paper we examine the effect of CX on the response to serum of several other cell cycle dependent genes, and extend the results with the mouse genes (21) to additional cell types.

We present here data on gene expression, measured as levels of specific cytoplasmic mRNAs. Possible mechanisms affecting these levels are discussed but not examined. Surprisingly, of the nine genes we and others have studied, none shows an inhibition of expression in the presence of CX. The mRNAs of all these genes are inducible in the complete absence of protein synthesis.

METHODS

Cells. tsAF8 cells were routinely grown at the permissive temperature of 34°C in Dulbecco's MEM with 10% calf serum. Cultures were made quiescent by two days incubation at 34°C in 0.5% calf serum, and were subsequently stimulated with 10% donor calf serum. Swiss 3T3 cells were cultured as described (26). Cells were continuously monitored for quiescence and stimulation by labeling with 0.2-0.5 μCi/ml [^{3}H]-methyl thymidine (NEN, 6.7 Ci/mmol) followed by autoradiography.

Cycloheximide Experiments. Cycloheximide was purchased from Calbiochem and was dissolved in Hanks' BSS. Swiss 3T3 cells were exposed to a final concentration of 25 μ g/ml in Dulbecco's MEM while tsAF8 cells were exposed to 50 μg/ml. In some experiments drug-treated and control cultures were labeled with [^{3}H]-leucine (Amersham, 50 Ci/mmol) at 0.5 μCi/ml. At various times after addition of the drug and [^{3}H]-leucine, the cultures were washed and scraped into 5% trichloroacetic acid. The cell pellets were held at 4°C overnight, washed with 1 ml trichloroacetic acid and dissolved in 0.1 N NaOH. Aliquots of this solution were counted to determine the effect of cycloheximide on protein synthesis. Protein synthesis, as measured by this technique, was inhibited by 99% in Swiss 3T3 cells, and by 97% in tsAF8 cells. In other experiments the RNA was isolated from drug-treated or control cells, and analysed as described below.

Plasmids. Plasmids used as probes were prepared either by cesium chloride centrifugation or chromatography on Sepharose 2B. pJE-3 and pKC-1 were isolated by Cochran et al. (21); p2A9, p2F1, and p4F1 are hamster cDNAs described by Hirschhorn et al. (22); pp53-271, the probe for p53, was a gift from Dr. Moshe

Oren (27); and pF0422, containing the human histone H3 gene, was a gift of Dr. Gary Stein.

Northern Blots. RNA was isolated from cultured cells as described (22). RNA concentrations were spectrophotometrically determined, and equal quantities of RNA were fractionated on 1% agarose gels containing 6.6% formaldehyde (22). The separated RNAs were subsequently transferred to nitrocellulose, and hybridized (28) to nick-translated (29) probes. All other conditions were similar to those in reference 22.

RESULTS

The genes investigated in the present experiments can be divided into four groups: 1) mouse sequences JE-3 and KC-1 inducible early in G_1 by platelet-derived growth factor (PDGF); 2) Syrian hamster sequences 2F1, 4F1 and 2A9, inducible early in G_1 by serum; 3) histone H3 gene, expressed in late G_1 or S phase; and 4) the cell cycle-dependent oncogene, p53. Non cell cycle-dependent genes were used as control.

Mouse Genes Inducible by PDGF. The cDNA clones, JE-3 and KC-1, (21) isolated from Balb/c 3T3 cells are induced by PDGF, even in the presence of CX (21). Indeed, CX itself increases their level of expression even in G_0 cells not stimulated to proliferate (21). We have confirmed these results in Swiss 3T3 cells where, in fact, the magnitude of the CX effect on the expression of JE-3 and KC-1 was the same in G_0 as in serum-stimulated cells (data not shown). Similar results were obtained with tsAF8 cells, Fig. 1 shows the effect of CX on the expression of KC-1 in G_0 and serum-stimulated tsAF8 cells. Both exposure to CX and serum stimulation lasted 4 hr. KC-1 expression is very low in hamster cells and cannot be detected in 15 μg of quiescent, or even stimulated cell total RNA (lanes 1 and 3). However, the induction of this mRNA by CX is such as to make it easily detectable (lanes 2,4). Clearly, KC-1 expression after serum stimulation is not inhibited by CX which by itself, causes an increase in mRNA levels. Similar results were obtained for JE-3 (Fig. 2), although in this case the expression is the absence of CX is also high.

Hamster Genes Inducible by Serum. 2F1, 4F1 and 2A9 are cDNA clones selected by differential screening of a cDNA library prepared from ts13 cells (22). The levels of mRNA's corresponding to the inserts of these clones increase in ts13 and tsAF8 cells stimulated by serum at the permissive temperature of 34°C (22).

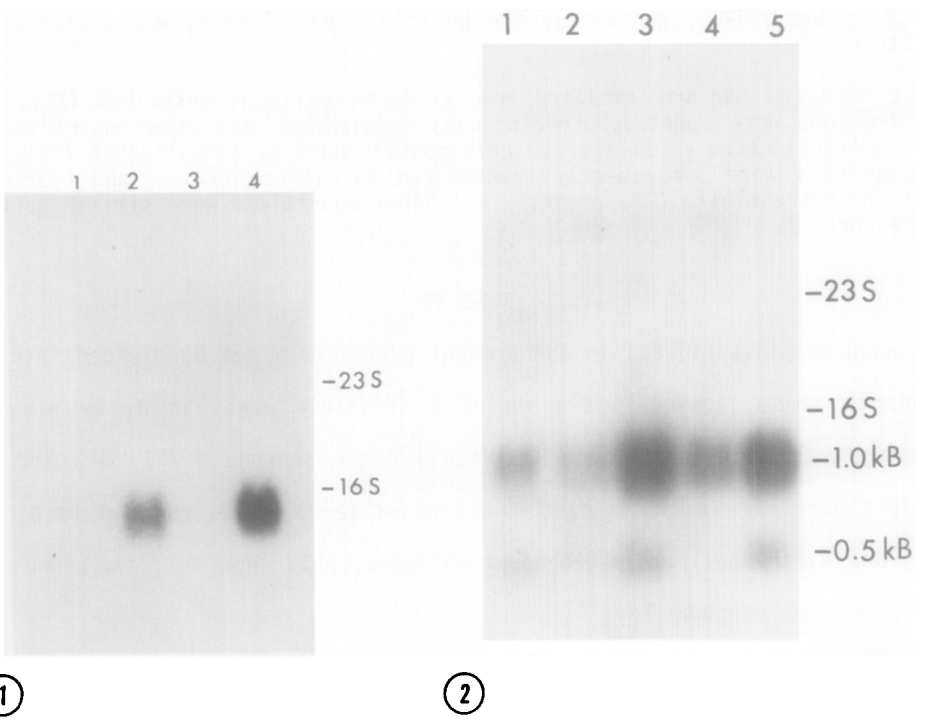
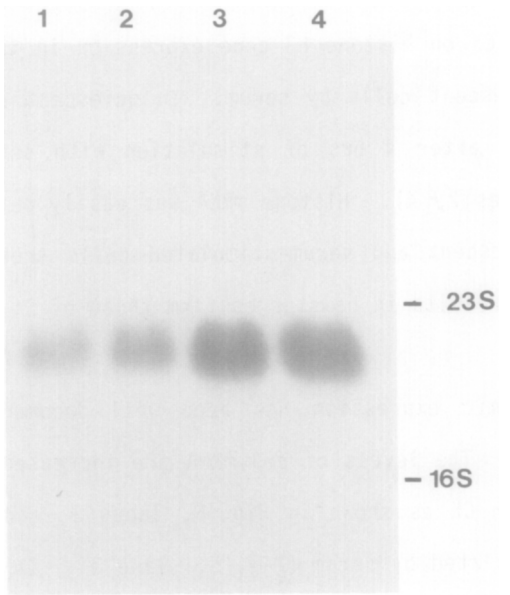


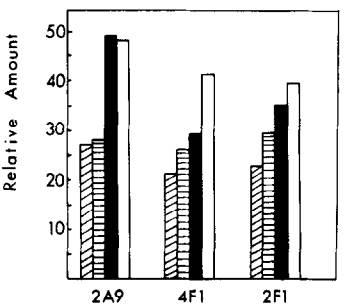
Fig. 1: KC-1 Expression in Serum-Stimulated and Cycloheximide Treated tsAF8 Cells. tsAF8 cells were plated and made quiescent as described in Methods. At time zero, cells were treated with cycloheximide, 50 µg/ml, either in medium containing 0.5% serum, or in the presence of 10% donor calf serum. Control cultures were either unstimulated, i.e., left in 0.5% serum, or stimulated with 10% serum in the absence of cycloheximide. Four hours later, the cells were harvested and RNA was extracted. Fifteen µg of cytoplasmic RNA were fractionated on 1% agarose gels containing 6.7% formaldehyde; these RNAs were subsequently transferred to Gene Screen, and hybridized to nick-translated KC-1 plasmid according to the manufacturer's instructions. The resulting autoradiogram is shown. Lane 1: quiescent cell RNA; lane 2: RNA from quiescent cells treated with cycloheximide; lane 3: RNA from cells stimulated with 10% serum; and lane 4: RNA from cells stimulated with 10% serum in the presence of cycloheximide. The positions of E. coli ribosomal 23S and 16S RNA are indicated.

Fig. 2: JE-3 and Histone H3 Expression in Serum-Stimulated and Cycloheximide Treated tsAF8 Cells. Cells were treated with cycloheximide with or without serum stimulation, as described in the legend to Fig. 1. RNA was extracted, fractionated on a 1% agarose gel containing 6.7% formaldehyde, and transferred to Gene Screen. The resulting blot was hybridized to a mixture of ^{32}P labeled JE-3 and histone H3 sequences, and the resultant autoradiogram is shown. Each lane contains RNA extracted from: 1) exponentially growing cells; 2) quiescent cells; 3) quiescent cells treated with 50 µg/ml cycloheximide for 4 hr; 4) cells stimulated with 10% serum for 4 hr; 5) cells stimulated with 10% serum for 4 hr in the presence of 50 µg/ml cycloheximide. The positions of JE-3, at 1.0 kB, and histone H3, at 0.5 kB, are indicated, as are the positions of 16S and 23S E. coli ribosomal RNAs.

The expression of 2F1 and 4F1 is increased earlier than the expression of 2A9, but all three return to G_0 levels by the time the cells reach S phase (22).



(3)



(4)

Fig. 3. 4F1 Expression in Serum Stimulated and Cycloheximide treated tsAF8 cells. Conditions and lanes as in Fig. 1. The positions of E. Coli ribosomal 23S and 16S RNA are indicated.

Fig. 4: Effect of Cycloheximide on the Expression of 2A9, 4F1 and 2F1 in tsAF8 Cells. Cells were treated and RNA extracted as described in the legend to Fig. 1. Fractionated RNAs were blotted onto Gene Screen and hybridized to ^{32}P labeled 2A9, 4F1, or 2F1 cDNAs. Densitometer readings of the corresponding autoradiograms are shown. In each case, the diagonally striped bar represents the RNA levels in quiescent cells; the horizontally striped bar, quiescent cells treated for 4 hr with cycloheximide at 50 μg/ml; the solid bar, cells stimulated for 4 hr with 10% serum; and the open bar, cells stimulated for 4 hr with 10% serum in the presence of 50 μg/ml cycloheximide.

Fig. 3 shows the expression of 4F1 in quiescent and stimulated cells treated with CX. While CX by itself induces only slightly the expression of this gene (compare lanes 1, 2), the drug has little effect on the induction by serum (lanes 3, 4).

Fig. 4 summarizes in graphical form the effects of CX and serum on the expression of these genes in tsAF8 cells. Unlike KC-1 and JE-3 whose expression is increased manyfold by CX, the expression of 2A9 and 2F1 is affected only slightly, if at all, by CX: 2A9 is totally unaffected while the expression of 2F1 increased 10-20% in the presence of the drug. While the effect of CX on the expression of these genes is not as dramatic as with the mouse genes, it is clear that no inhibition of specific mRNA induction by serum

occurs. Similar results were obtained with Swiss 3T3 cells. (data not shown).

Histone. Fig. 2 shows the effect of CX on histone H3 gene expression in tsAF8 cells early after stimulation of quiescent cells by serum. In quiescent cell RNA, or in RNA extracted from cells after 4 hrs of stimulation with serum, histone H3 mRNA was undetectable (lanes 2, 4). Histone mRNA was easily detectable, however, in RNA from both quiescent and serum-stimulated cells treated with CX for 4 hr (lanes 3, 5). These results in hamster confirm those of Stimac et al. (30) in mouse cells.

Oncogenes. The effect of CX on c-myc expression has been well documented (5,25) and will not be repeated here. The levels of p53 mRNA are increased in quiescent Swiss 3T3 cells treated with CX as shown in Fig. 5, lanes 1, and 2, although not as much as in cells stimulated by serum (Fig. 5, lane 3). CX is, moreover, unable to inhibit the induction of p53 mRNA by serum (compare Fig. 5, lanes 1 and 4). The mouse p53 probe used does not cross react with hamster mRNA, so we were unable to extend these results to tsAF8 cells.

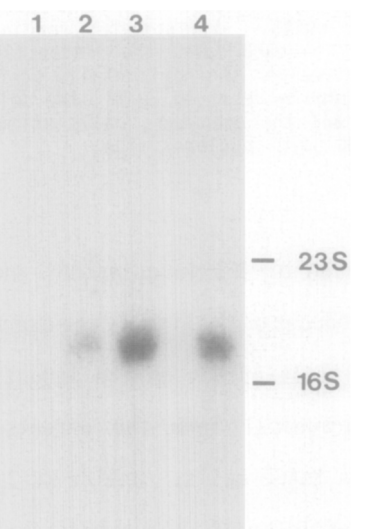


Fig. 5: Effect of Cycloheximide on the Expression of p53 in Swiss 3T3 Cells. Swiss 3T3 cells were plated and made quiescent, as described in Methods. At time zero, the cells were treated with 25 μg/ml cycloheximide, 10% serum, both or neither, and 6 hr later RNA was extracted. These RNAs were fractionated on 1% agarose formaldehyde gels, blotted onto nitrocellulose and hybridized to nick translated p53 cDNA. Lane 1: quiescent cells; lane 2: cells treated with 25 μg/ml cycloheximide; lane 3: cells stimulated with 10% serum; lane 4: cells stimulated with 10% serum in the presence of 25 μg/ml cycloheximide.

Non Cell Cycle-Dependent Genes. We have also investigated the behavior of sequences whose expression is not cell cycle dependent. The mRNAs corresponding to these cDNAs are not affected by CX (data not shown).

DISCUSSION

The effect of CX was investigated on the presumption that inducibility of a gene in the presence of the drug would indicate that the increase in RNA levels does not require previous protein synthesis. In other words, genes whose serum inducibility is not inhibited by CX may represent the cells' primary response to the growth factors in serum. By this logic, c-myc, p53 and the five G_1 specific genes (JE-3, KC-1, 2F1, 4F1 and 2A9) represent part of the primary response of cells to serum and its growth factors. But, histone H3 expression is also increased by treatment with CX, a result which is in agreement with other reports (30,31). Core histones, however, are not induced in G_1 ts mutants stimulated by serum at the nonpermissive temperature (4,32), indicating that they are not part of the primary response to serum. Moreover, Sive et al. (31) and Stimac et al. (30) show that the increase in histone mRNA concentration in cycloheximide treated cells is due to increased mRNA stability.

Thus, there may be alternative mechanisms to explain the induction of mRNAs in the presence of CX. These mechanisms include: 1) increased stabilty, as discussed above, or 2) repression by a labile protein, which is degraded and not resynthesized in the presence of CX, and in whose absence gene transcription is maximal. Serum growth factors may displace this repressor protein, which would explain the action of both serum and CX. It may be that more than one mechanism is operative, since the genes we have examined may be divided into two groups based on their inducibility by CX: 1) super induction, as in the case of JE-3, KC-1 and p53, where the levels of the specific mRNAs are increased drastically in the presence of CX over those in control cultures, and 2) normal induction, as in the case of 2F1, 2A9, and 4F1 in which CX does not affect the normal induction of these mRNAs by serum. The first group may be regulated by a labile repressor, while the second may be stabilized, but additional data are required to distinguish these possibilities.

In any case, it is clear that we are unable to use the results with cycloheximide to distinguish genes which represent a cell's primary response to growth factors.

The most striking result of the data presented here is that of the known cell cycle-dependent genes discussed here, all are fully expressed, or even overinduced in the absence of protein synthesis. These include KC-1, JE-3, 2F1, 4F1, 2A9, p53, c-myc, (5) histone H3, and c-fos (12). This insensitivity to protein synthesis inhibition may be characteristic of many cell cycle dependent genes, and may reflect a common mechanism regulating the expression of such genes.

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