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**Butyrate selectively activates the metallothionein gene in teratocarcinoma cells and induces hypersensitivity to metal induction**

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ABSTRACT

The expression of metallothionein genes (MT-I and MT-II) was shown to be enhanced within 2 h of addition of 2.5-5 mM sodium butyrate to cultures of teratocarcinoma cells. Both undifferentiated stem cells (F9 and OC15) and differentiated cells (PSA5E and OC15 END) reacted similarly to butyrate by increased accumulation of MT mRNAs. As expected, all of the teratocarcinoma cells that were tested also responded to $Zn^{2+}$ and $Cd^{2+}$ by 5- to 10-fold increases in MT mRNA accumulation within 2-24 h of metal addition to the culture media. Surprisingly, MT genes in cells pretreated with butyrate were hypersensitive to metal induction, and this was demonstrated by accumulated transcript levels and by synthesis of MT protein. The maximal metal response was obtained by exposure of cells to butyrate for around 5-8 h together with 10 μM heavy metals. Metal additions to culture media over a range of concentrations and times only induced half the levels of MT mRNA that were achieved by butyrate plus metals. Butyrate enhanced the rate of accumulation of MT mRNA in response to metals, increased the sensitivity of the MT gene to metals, and protected cells from toxic effects of high concentrations of metals. The butyrate and metal ion responses were selective in that no accumulation of c-*myc*, c-*fms*, HSP-70, or AFP mRNA was detected. However, c-*fos* mRNA accumulated in cells exposed to toxic concentrations of metals (50 μM and higher) and this was also potentiated by butyrate treatment. These results suggest that butyrate alters the chromatin conformation of both the MT-I and MT-II genes leading to an accentuated transcriptional response to metals.

## INTRODUCTION

Sodium butyrate has a variety of effects when added to the medium of cultured cells. It is a potent inducer of erythroid differentiation (1) and can activate specific genes in a variety of cell types (2,3). Certain teratocarcinoma stem cells (embryonal carcinoma) are weakly stimulated to differentiate by butyrate (4). In contrast, sodium butyrate inhibits the differentiation of F9 embryonal carcinoma cells along both major pathways to parietal (5) and visceral endoderm (6). F9 cells stimulated to differentiate by retinoic acid in the medium are almost completely inhibited if 2.5-5 mM sodium butyrate is also present. Levine et al. (5) showed that at least 2 genes (laminin and type IV collagen) normally activated by retinoic acid are specifically

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repressed by butyrate. It was suggested that hyperacetylation of histones could account for this effect since butyrate represses the activity of the histone deacetylase enzyme. The end result is an altered structure of chromatin which could have rather non-specific effects on gene expression. We described previously the regulation of the MT gene during the development of the mouse as well as in teratocarcinoma cells stimulated to differentiate with retinoic acid (7). In light of the disparate effects of butyrate on embryonal carcinoma cells, we investigated whether this chemical could affect the activity of the metallothionein genes (MT-I and MT-II) in murine teratocarcinoma cells, both undifferentiated and differentiated.

The MT genes in a variety of cell lines and in liver and kidney of whole animals respond to heavy metals such as $Zn^{2+}$, $Cu^{2+}$, and $Cd^{2+}$ by a rapid stimulation of transcription rate (8-12). Glucocorticoids can also increase MT synthesis in liver, hepatocytes, and many other cell types (11,13,14,15,16). In addition, MT genes can be induced by a variety of acute stresses such as bacterial lipopolysaccharide and interleukin-1 (10) and also by interferons (17,18), ultra-violet (19), and X-ray irradiation (20), although the mechanisms are obscure. The 5′ flanking DNA of the MT gene contains sequences that are required for induction by metals, although the precise mode of activation is not yet known.

What then are the roles of these multi-gene families that allow for their activation by a variety of agents? The precise functions of the two main isoprotein forms, MT-I and II, are unknown, but since heavy metal ions are bound with high affinity, they may play roles in zinc and copper homeostasis and metal detoxification. It has been suggested that since MT expression accompanies some stress states, there may be a role for MT in biological responses to a variety of stresses (21).

The metallothionein gene is highly active during the gestation period in mouse, with maximum expression between days 12 and 15 in fetal liver and certain extraembryonic tissues (7). We noted previously that basal levels of expression of MT are high in parietal endoderm and visceral yolk sac endoderm but low in other extraembryonic tissues such as amnion and placenta. We also observed high expression in some but not all teratocarcinoma stem and differentiated cell lines (7), and this suggested that the MT gene may be active early in embryonic development.

The MT promoter placed in a 5′ position with DNA sequences for several genes has been used to construct transgenic mice after microinjection into

mouse eggs (22-25). It is therefore useful to understand the factors that can regulate exogenous genes that are under the control of the MT promoter. Similarly, the MT promoter has been used to influence the expression of exogenous genes transfected into cultured cells in studies to map DNA regulatory sequences and tissue-specific activating factors. We undertook, therefore, a study of some of the factors which regulate endogenous MT gene expression in cell lines and especially in embryonic cell lines.

Basal levels of MT expression in teratocarcinoma cells do not correlate with the degree of differentiation between different cell lines, and they are also somewhat variable. We sought to determine the external signals that induce MT expression and whether these signals act directly on the gene or are mediated by secondary processes. We showed earlier that retinoic acid is one modulator of MT expression, although in the case of F9 cells the effect appears to be indirect, since several days elapse and differentiation occurs before basal MT expression is elevated (7). The effect of heavy metals on MT levels in teratocarcinoma cells has not been reported, and we describe here the nature of the effects of metals and butyrate on MT transcript levels in these cells. We show here that sodium butyrate not only induces significant elevation of MT mRNA and protein levels in two kinds of teratocarcinoma cells, but it also renders the cells hypersensitive to metal ion induction. These findings may be useful in studies of regulatory mechanisms that operate not only on endogenous genes but also on transfected genes spliced to the MT promoter/enhancer in embryonal carcinoma and in developing embryos.

## METHODS

### Cell Culture

Embryonal carcinoma cells F9, PC13, and OC15S1 were maintained in culture as described before (26,27). OC15 cells were passaged every 2 to 3 days to prevent spontaneous differentiation. To stimulate the differentiation of OC15 EC cells into OC15 END, cells were seeded at $10^6$/100 mm dish in medium containing 1 $\mu$M retinoic acid for 4 days with refeeding on day 2. PSA5E is a differentiated endoderm-like cell line (28) that expresses some of the characteristics of visceral endoderm. PSA5E cells were exposed for short periods of time to 5 mM sodium butyrate added from a sterile stock of 0.25 M in water adjusted to pH 7.4. For controls in some experiments, 5 mM sodium acetate or sodium propionate was added. For overnight incubations, sodium butyrate was reduced to 2.5 mM. A mixture of $Cd^{2+}$ and $Zn^{2+}$ was used at 5 $\mu$M. EC cells

were cultured with retinoic acid for the times indicated to stimulate differentiation as described before (7). Usually ten 100 mm dishes were used to extract RNA from cell pellets.

Radiolabelling

Sets of 35 mm dishes that contained subconfluent cultures of OC15 EC cells or PSA5E cells were tested for biosynthesis of metallothionein protein. Monolayers treated for the times indicated in the text were washed, preincubated in cysteine-free medium (Swims, Flow Laboratories) containing 10% dialyzed fetal bovine serum. After 1 hr, fresh cysteine-free medium (0.5 ml) containing 10% dialyzed fetal bovine serum, butyrate, 5 $\mu$M $CdCl_2$ plus 5 $\mu$M $ZnCl_2$ where appropriate, and 20-50 $\mu$Ci per ml of $^{35}$S-cysteine was added to each dish for a period of 3 hr. Medium was removed, cell layers washed in warm PBS, and 0.1 ml of lysing medium was added (lysing medium = 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM dithiothreitol, and 1% Nonidet 40). Lysates were centrifuged at 15,000 g for 15 min at $4^{o}$. Twenty $\mu$l of each lysate supernatant was removed to a separate Eppendorf tube, and processed as described (29). Equal amounts of radioactivity were loaded onto 20% polyacrylamide gels containing SDS (30) and electrophoresed until the tracking dye had passed off the end of the gel. Labelled metallothionein is recognized on fluorographs prepared from the fixed and dried gels as the fastest moving radioactive protein. Since metallothionein is a very small peptide (6000 daltons) and contains 30% cysteine, these characteristics enable it to be readily detected.

Hybridization Probes

Mouse MT-I and MT-II cDNA clones were kindly provided by Dr. Richard Palmiter (Seattle, WA). The mouse AFP cDNA clone was described previously (7). Mouse c-*fos* and c-*myc* probes were from the American Type Culture Collection, Maryland, and mouse c-*fms* was donated by Dr. C. H. Sherr (31). Mouse heat-shock protein HSP-70 probe was from Dr. R. Morimoto, Northwestern University. The cDNA inserts were subcloned into the SP6 vectors (Promega Biotech, Madison, WI) and used as templates for the synthesis of $^{32}$P-labelled RNA probes (32). Probes had a specific activity of about 5 x $10^8$ dpm/$\mu$g.

RNA Extraction

Total RNA was isolated from cell pellets by sodium dodecyl sulfate (SDS)-phenol extractions (33) and precipitated with 3 M ammonium acetate (34) to precipitate RNA from the soluble DNA.

Northern Blot Analysis of RNA

Northern blot analysis was accomplished by electrophoresis of RNA (7 $\mu$g) in 1.5% agarose gels containing 2.2 M formaldehyde (35) with constant recircu-

lation of the running buffer (2.2 M formaldehyde, 0.018 M $Na_2HPO_4$, 0.002 M $NaH_2PO_4$). Gels were soaked twice for 15 minutes each in 10 mM sodium phosphate buffer, pH 7.0, and blotted to nitrocellulose according to the procedure of Thomas (36). The integrity of the RNA was monitored by acridine orange staining of gels. Prehybridization, hybridization, and posthybridization treatments of filters were as described previously (7). Quantitation of RNA levels on blots was done in two ways, densitometric scanning of autoradiographs as described before (37) and by counting the excised radioactive RNA/RNA hybrids in a toluene-based fluor in a scintillation counter.

## RESULTS

The basal levels of MT mRNA in embryonal carcinoma cell lines are different for each cell line. The F9 cell line has intermediate basal levels of MT expression, while a similar line, PC13, has very high levels (7). On the other hand, OC15S1 stem cells usually have very low basal levels. We therefore chose the latter cell line for most of the studies here. OC15 embryonal carcinoma cells differentiate during 4 days of culture in the presence of retinoic acid to larger, flatter endoderm-like cells, and these, together with another early embryonic endoderm-like teratocarcinoma cell line, PSA5E, form the framework for these studies.

### Butyrate Affects the Metallothionein Gene Rapidly and Specifically

Some early results showed that OC15 EC cells exposed to 2.5 mM sodium butyrate overnight had 7- to 20-fold increased levels of MT mRNA (not shown). Slight changes in cell morphology observed as increased size and flattening of cells indicated that differentiation may be stimulated by butyrate. We therefore analyzed the MT mRNA levels in both OC15 undifferentiated cells (EC) and cells produced after 4 days of exposure of OC15 cells to 1 $\mu$M retinoic acid (OC15 END). Cells were harvested after various times of exposure to butyrate, and the RNA was analyzed by Northern blots and quantified by densitometry as described in the Methods section. Figure 1 shows that both undifferentiated OC15 EC and differentiated END cells respond similarly and rapidly to butyrate. For OC15 EC cells, averages of three experiments are shown in Figure 1 to demonstrate that there was some variability in mRNA levels. In two out of four experiments, a response to butyrate was seen after 2 h of exposure (inset) as 4-fold higher levels of MT mRNA and this increased to 9-fold higher after 4 h. In two experiments the response was delayed slightly. F9 EC cells responded similarly and rapidly but started at a higher basal level (data not shown). OC15 END cells also started at a higher basal level, and stimulated levels were

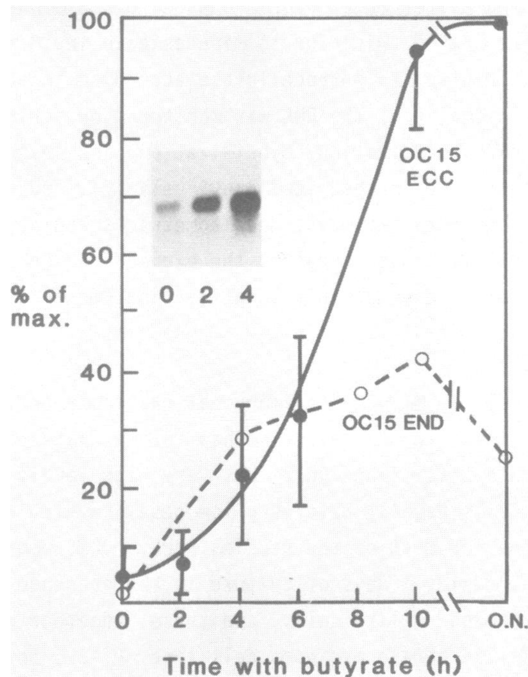


Figure 1. Changes of MT mRNA levels with time of exposure to butyrate. RNA extracted from OC15 EC cells and differentiated OC15 END after various times of exposure to butyrate were analyzed. Northern blots were quantitated by densitometric scanning (see Materials and Methods) and values are expressed as a percentage of the maximum level of MT mRNA reached. Inset shows Northern blots produced after 0, 2, and 4 h of stimulation of OC15 EC cells with butyrate. This experiment shows that butyrate stimulated MT mRNA levels within 2 h of addition to the medium.

seen at 4 h, the earliest time examined (Fig. 1). Exposure of cells to butyrate for 10 h was sufficient for maximal responses.

The effect of butyrate on MT induction in teratocarcinoma cells was specific for this reagent since sodium propionate and sodium acetate had no effect. We also assayed for other gene products during butyrate treatment and found no expression of alphafetoprotein mRNA in F9 EC cells and no stimulation of c-*myc*, or c-*fms* oncogenes and little change in expression of c-*fos*, heat-shock protein, HSP-70, in both OC15 EC cells and PSA5E cells (data not shown).

Butyrate Induces Hypersensitivity to Metal Ions

Both butyrate and heavy metal ions are potent inducers of MT gene expression on OC15 EC cells (Fig. 2A) and in PSA5E cells (Fig. 2B). Butyrate and metal ions together induced even higher levels of MT which appeared to be

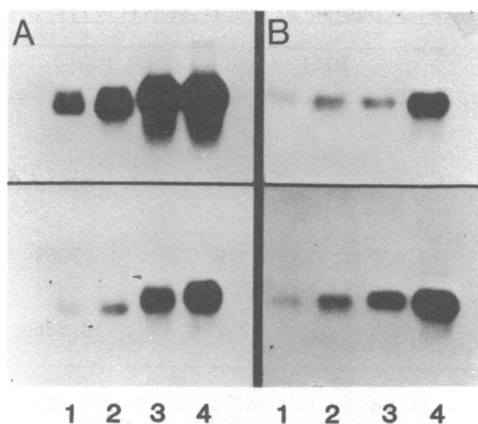


Figure 2. The effect of heavy metals and butyrate on teratocarcinoma cells. Northern blot analysis of accumulated RNA in OC15 EC cells (A) and PSA5E cells (B). $^{32}P$-labelled riboprobes applied were MT-I (upper panel) and MT-II (lower panel). Lanes 1, control cells without drug treatment; lanes 2, cells treated with butyrate for 7 h; lanes 3, cells treated with metals for 2 h; lanes 4, cells treated with both metals and butyrate.

greater than additive in OC15 EC (undifferentiated) cells (Fig. 2A, lane 4) but was more dramatic in PSA5E (differentiated) cells (Fig. 2B, lane 4).

In order to determine if the effect of butyrate and metals on MT expression was additive or synergistic for OC15 EC cells, the RNA/RNA spots were cut from nitrocellulose sheets and counted. Table 1 shows that after 7 h of butyrate treatment, the rapid (2 h) response of the cells to $Cd^{2+}$ and $Zn^{2+}$ was doubled in the case of MT-I and more than tripled for MT-II. The 7 h exposure of OC15 EC cells to butyrate alone in this series of experiments was submaximal for stimulation of MT expression (2.1-fold stimulation for MT-I), but this period gave maximal sensitivity to induction by metals (7.2-fold). In the case of MT-I, the combined effect of butyrate and metals was additive while for MT-II, the induction by metals was more than additive with butyrate. This is seen also in Figure 2A, but in this case, the autoradiograph is over-exposed to show the expression of MT by control cultures without drugs. When all measurements of MT-I and II induction were averaged, co-cultivation of OC15 cells in butyrate plus metals gave $1.81 \pm 0.56$ ($n = 10$)-fold higher levels of mRNA than the <u>added</u> values for separately stimulated cultures. For PSA5E cells, synergism between metal and butyrate stimulation was similar to that observed for OC15 EC cells; together these chemicals induced $1.73 \pm 0.15$ ($n = 9$)-fold greater levels of MT-I and MT-II mRNA than in the added values for separately stimulated cultures.

Table 1. Butyrate induction of metal sensitivity in OC15 EC cells

| Additions | MT-I cpm | MT-I increase | MT-II cpm | MT-II increase |
|---|---|---|---|---|
| None = Control | 3511 | 1.0 | 805 | 1.0 |
| Butyrate (5 mM, 7 h) | 7289 | 2.1 | 1019 | 1.3 |
| Metals (5 M, 2 h) | 13,421 | 3.8 | 3059 | 3.8 |
| Butyrate + Metals | 25,125 | 7.2 | 10,886 | 13.5 |

OC15S1 stem cells were grown to subconfluence before the addition of sodium butyrate (5 mM) for 7 h and/or $CdCl_2$ and $ZnCl_2$ (5 µm) for the last 2 h as indicated. Cultures were harvested and RNA isolated as described in the Methods section. After electrophoresis, total RNA (7 µg each sample) was blotted onto nitrocellulose and $^{32}$P-labelled metallothionein probes used to detect MT mRNA. The labelled RNA hybrids were located using the autoradiograph and were excised and counted. Two similar experiments were averaged to give the values in Table 1.

The response of the MT genes to butyrate and metal induction was also studied at the protein level. Presumably, MT protein is needed to react with the excess of metal ions reaching the cells from the medium. Interestingly, although 3 h exposure of OC15 EC cells to metals induced 16-fold higher levels

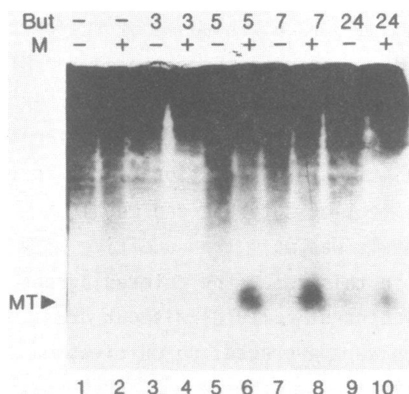


Figure 3. Biosynthesis of metallothionein protein in OC15 cells.
OC15 cells were treated with 5 mM sodium butyrate for the times indicated (in hours) and with heavy metal ions (M) for the duration of the 3h labelling period. An equal amount of radioactive protein (2 x $10^4$ cpm) for each sample was analyzed (see Materials and Methods for other details). MT marks the position of metallothionein protein.

of mRNA (see Figure 4 below), MT protein was not detected on the fluorograph shown in Figure 3, although over-exposure revealed a small amount (Figure 3, lane 2). Figure 3 shows that 3 h of exposure of OC15 EC cells to butyrate even with 3 h of metal induction was also insufficient to produce readily detectable amounts of protein (lanes 3+4). The presence of butyrate for 5-7 h achieved a large increase in the induction of MT protein by $Cd^{2+}$ and $Zn^{2+}$ in OC15 EC cells (lanes 5-8). By 24 h of butyrate treatment, the stimulation of MT protein by metals had declined somewhat (lanes 9 & 10). The potentiated synthesis of MT protein gives clear evidence that pre-exposure of cells to butyrate renders the MT gene hypersensitive to the inductive effect of metals (compare lane 2 with lane 6 or 8 in Figure 3).

We examined the kinetics of the butyrate effect on metal responsiveness of the MT gene in two further ways. First, we determined the maximal exposure to butyrate needed to produce maximal accumulation of MT transcripts in response to metals at 5 µM for 2 h. In both OC15 and PSA5E cells, the highest levels of MT mRNA detected by Northern blotting and scanning densitometry was after 4 to 7 h exposure to butyrate (data not shown). This result confirmed the protein synthesis data above. Second, we fixed the time of exposure to

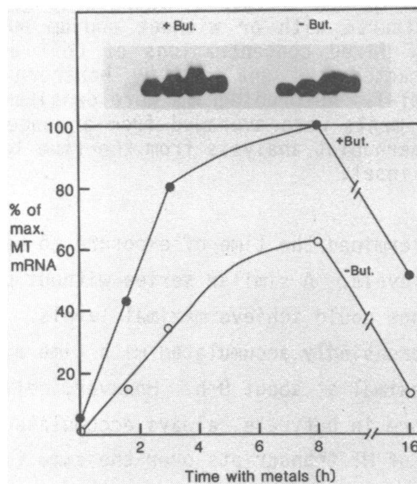


Figure 4. Stimulation of MT mRNA levels by increasing time of exposure to heavy metal ions. Cd + Zn at 5 µM were added to cells that were also incubated with or without butyrate (5 mM) for 6 to 8 h. RNA was prepared at the indicated times and analyzed for MT transcripts by Northern blotting (see inset). Several exposures of the fluorograms were analyzed by densitometry to compute levels of MT mRNA.

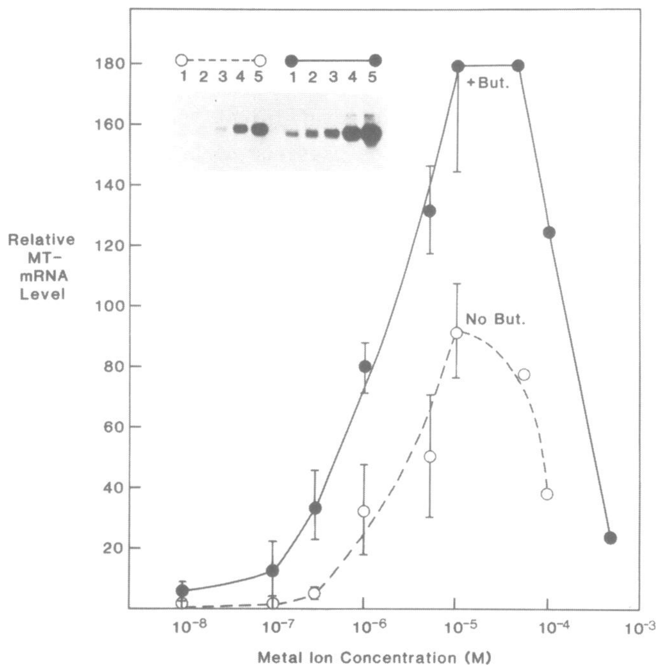


Figure 5. Stimulation of MT mRNA levels by increasing metal ion concentrations. OC15 EC cells were cultured with or without sodium butyrate (5 mM) for 4 h before the addition of fixed concentrations of $Cd^{2+}$ and $Zn^{2+}$ for 2 further hours. RNA was extracted and analyzed by Northern blotting (inset) for accumulated levels of MT-I. Autoradiograms were densitometrically scanned, and results of three experiments were averaged from a range of different exposure times. A typical Northern blot analysis from the five lowest concentrations of metals is shown in the inset.

butyrate at 6 h and determined the time of exposure to metals needed to achieve maximal MT transcript levels. A similar series without butyrate was used to determine if metals alone could achieve maximal levels. Figure 4 shows that MT transcripts were increasingly accumulated with time of exposure of cells to 5 μM metals and were maximal at about 8 h. However, cells which had been pre-incubated for 6 h or more in butyrate, always accumulated greater levels (approximately 2-fold) of MT transcripts over the same time course of exposure to metals alone. In other words, metals alone could not achieve the very high levels obtained when butyrate was also present.

We then asked if the concentration of metals used was sufficient to achieve maximal MT induction and whether this concentration was different in the presence of butyrate. This was studied by fixing the time of exposure to

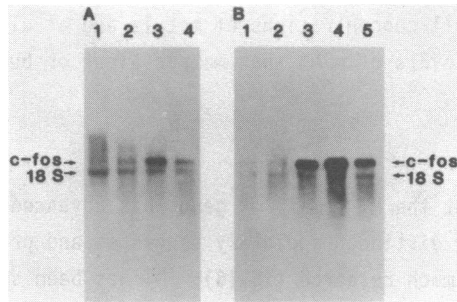


Figure 6. The stimulation of c-fos by toxic levels of metals in OC15 EC cells. Northern blot analysis of total RNA extracted from cells untreated (A) or treated (B) with 5 mM butyrate for 4 h before further incubation with Zn plus Cd for 2 h. Lane 1, $10^{-6}$ M; lane 2, $10^{-5}$ M; lane 3, 5 x $10^{-5}$ M; lane 4, $10^{-4}$ M; lane 5, 5 x $10^{-4}$ M metals. c-*fos* mRNA (2.2 kb) is indicated above 18s rRNA which has artifactually absorbed some labelled probe. Note that butyrate enhances c-*fos* induction.

metals at 2 h and to butyrate at 6 h and varying the concentration of metals. Figure 5 shows that the accumulated levels of MT mRNA were two- to three-fold higher in the presence of butyrate at all metal concentrations, while the maximal level was reached using 10 μM metals both in the presence and absence of butyrate. In addition, the presence of 5 mM butyrate for 6 h increased the response to metals at very low concentrations (0.1 μM) and reduced the concentration of metals required for half-maximal response to 1.3 μM from 2.4 μM in the absence of butyrate. At concentrations of metals higher than 10 μM, OC15 EC cells accumulated lower levels of MT mRNA (40% of maximal with 100 μM metals) and butyrate was partially able to protect the cells from these toxic levels probably by maintaining higher absolute levels of MT protein (Figure 3). Cells incubated in 500 μM metals for 2 h had all detached from the dish and were probably undergoing lysis.

Interestingly, at toxic levels of metals, OC15 EC cells accumulated high levels of c-*fos* mRNA, and this was also potentiated by butyrate (Figure 6). The maximal responses of the c-*fos* gene were seen at 50 μM metals, or 100 μM metals in the presence of butyrate (Fig. 6, lanes 4).

In summary, both metals and butyrate markedly stimulated the accumulation of MT mRNA levels after 2 h of exposure of undifferentiated and differentiated teratocarcinoma cells. Longer exposures of both chemicals were needed to affect MT protein levels, but butyrate present for 5-7 h dramatically hypersensitized the induction of MT protein by metals. Pre-incubation of teratocarcinoma cells with butyrate for 6-24 h increased the responses of the MT gene to

metal induction at all concentrations of metals and at all exposure times, thus producing elevated levels of mRNA that metals alone or butyrate alone could not attain.

## DISCUSSION

Knowledge about the MT family of genes has advanced greatly in the last few years, and their distinct regulatory sequences and promoter elements have been the subject of much research (15,16). MT has been suggested as playing a role in growth control in neoplasia (21), as well as in developing tissues because of its dramatic changes in expression during the gestation period. We have studied some additional factors that affect MT expression in cells equivalent to primitive ectoderm (4th-7th day) and to their extraembryonic endoderm products in order to make suggestions about how the gene may be regulated as well as how MT genes may become induced during development.

We chose a drug that is known to affect the structure of chromatin and that has known inductive effects on certain characteristic genes. Sodium butyrate is a potent inducer of erythroid differentiation (1) but inhibits the differentiation of F9 embryonal carcinoma cells (5,6). The induction of at least three genes in F9 cells is selectively repressed by butyrate, namely, laminin, type IV collagen, and AFP (5,6). Here we show that the metallothionein gene is distinct in being induced up to 20-fold by butyrate, and, as early as 2 h after treatment of OC15 EC cells, 4-fold increases in MT mRNA can be detected. We have not attempted to measure transcription rates in this study, but the rapidity of response suggests that there may be a direct effect on the MT gene to stimulate transcriptional rate. Birren and Herschman (38) demonstrated that a rat hepatoma cell line was also stimulated to respond similarly to butyrate and with similar kinetics. They showed that butyrate stimulated MT-1 transcription in the hepatoma line and that butyrate and dexamethasone activated MT additively rather than synergistically. In the hepatoma line as in F9 cells, butyrate blocked the hormone-stimulated expression of other tissue-specific genes.

The production of more "open" chromatin thought to be induced by butyrate might be expected to activate a broad range of genes. This was not the case; butyrate did not activate any other gene that we tested, since AFP, c-*fos*, c-*fms*, c-*myc*, and HSP-70 all remained unchanged. Therefore, the action of butyrate is specific and because of its rapidity, and other evidence (5,38) is likely to be at the chromatin level.

A further clue to the mechanism of butyrate action lies in its ability to

increase the sensitivity of MT gene to induction by heavy metal ions. It has been shown previously that the mouse MT promoter is hypersensitive to nuclease digestion and that this region becomes increasingly sensitive following Cd induction (39). The MT-I gene in mouse lymphoid cells has been shown to be controlled by DNA methylation (40). These results imply that metals may act by loosening chromatin structure. Heavy metal ions induce MT gene activity through the mediation of a metal-responsive element in the gene (15). A butyrate-responsive element in the MT genes may be involved, but has not been identified yet. If it does exist, it accompanies the several kinds of DNA elements in the gene that have already been identified. We think that it is more likely to be a general effect such as loosening the chromatin structure of those genes involved in responses to stress such as MT and c-_fos_ (41). The induction of the c-_fos_ gene by toxic levels of metals and its further induction by butyrate (Fig. 6) support this suggestion.

The maximal levels of MT mRNA accumulated by teratocarcinoma cells even after induction by optimal exposure to heavy metals are lower than those achieved by butyrate plus metals (Figs. 2,3,4 & 5). This finding is consistent with the notion that butyrate stimulates the MT gene by a different mechanism to metals. Although a strong synergism is not obtained, the effect of butyrate with metals is greater than additive. At the mRNA level, we obtained 1.7 to 1.8-fold higher levels of MT mRNA in the presence of both chemicals compared to the added separate levels of each. The synthesis of MT proteins, however, was strongly synergized by butyrate with metals (Fig. 3) and this could indicate that butyrate may also act during the translation of MT mRNA. This has never been reported, but the possibility can be readily tested in a cell-free translation system. Meanwhile, we favor an explanation based on an effect on the chromatin structure of a restricted set of genes. Further support for the hypothesis arises from our studies that demonstrate that 5-azacytidine, an agent that affects the methylated state of DNA (42), also leads to hypersensitization of the MT gene to metals and to butyrate plus metals (data not shown).

Further investigations using butyrate as a selective gene inducer promise to be useful for understanding mechanisms of activation of endogenous MT genes. Even more significantly, the effect of butyrate on exogenous MT promoter sequences spliced together with other genes and introduced into cells may give some insights into the necessary regulatory sequences in the MT gene as well as into the roles of linked genes. This is a readily tested proposition. The transfected genes together with endogenous genes may be activated in several

stages (and possibly by different mechanisms for each agent), by butyrate alone, by metals alone and by both agents together.

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