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Activation of metallothionein expression is potentiated by DNA sequences present in the herpes simplex virus thymidine kinase gene

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A mouse cell line (Ltk– aprt–) which is resistant to the anti-viral effects of interferon also has a reduced ability to synthesize metallothionein on exposure to cadmium. Like the ability to respond to interferon, cadmium-induced metallothionein synthesis is restored to wild-type levels in clones obtained by introducing a thymidine kinase gene into Ltk– aprt– cells. Transfection of other genes does not have such an effect. Since metallothionein expression is also activated by interferon the results suggest that the regulation of several genes which are responsive to interferon can be modulated by specific sequences present in the Herpes virus thymidine kinase gene.

Metallothionein; Interferon; Thymidine kinase; Induction

1. INTRODUCTION

Metallothioneins are small, cysteine-rich polypeptides which are thought to be involved in cellular defense mechanisms [1]. Transcription of metallothionein genes is activated by exposure to heavy metals or to glucocorticoids [2–4] and also by interferon [5]. Upstream regulatory elements present in both the murine and human genes have been identified [6–8] and provide a model system for studying the regulation of gene expression.

We have previously shown that mouse Ltk– aprt– cells fail to develop an anti-viral state when treated with interferon, but cell clones derived from this line by introduction of a herpes simplex virus (HSV) thymidine kinase (tk) gene regain the capacity to activate an anti-viral state and synthesize specific enzymes on exposure to interferon [9,10]. We show here that Ltk– aprt– cells are also defective in their capacity to activate metallothionein expression when exposed to cadmium but that introduction of HSV tk gene sequences restores the inducibility of this gene.

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2. METHODS

2.1. *Cells and cell culture*

The origin of the Ltk– aprt– line is described elsewhere [10]. Cells were grown in minimal essential medium (Earle's salts) containing 10% heat-inactivated newborn calf serum, 100 U/ml penicillin and 50 μg/ml streptomycin. Transfections were performed as in [9] and tk(+) clones selected by growth in medium containing HAT (hypoxanthine, aminopterin and thymidine) or

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G-418 (Geneticin, Gibco-Biocult, Grand Island, NY).

2.2. *Metallothionein synthesis*

Sub-confluent cultures in 10 cm dishes were treated without or with cadmium sulphate (15 μM) for 4 h and then radiolabelled for 3 h with [^{35}S]cysteine (20 μCi/ml; 1205 Ci/mmol, Amersham, Arlington Heights, IL) in cysteine-free medium. Extracts were prepared and analysed by Sephadex G-75 (medium) chromatography [11]. Fractions (0.6 ml) were collected and 0.1 ml aliquots counted in Liquiscint (National Diagnostics, Somerville, NJ). Peak fractions were pooled and, after lyophilization, reduced and alkylated (10 mM 2-mercaptoethanol for 30 min followed by 10 mg/ml of iodoacetamide for 60 min). Aliquots corresponding to equal amounts of cellular protein were analysed by SDS-PAGE and autoradiography. Densitometry was performed with a Joyce-Loebl densitometer.

2.3. *Metallothionein mRNA levels*

Cells (approx. 1×10^7/10 cm dish) were incubated in medium containing 0 or 15 μM cadmium for the times indicated. Extracts for cytoblotting were prepared and blotted exactly as described [12]. For Northern blotting, extracts were prepared in 10 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 100 U/ml placental RNase inhibitor, centrifuged at 5000 $\times$ *g* for 5 min at 4°C and total cytoplasmic RNA extracted with phenol. After denaturation in formamide and formaldehyde, equal amounts of RNA were electrophoresed on 1.5% agarose gels, transferred to Nytran membranes (Schleicher and Schuell, Keene, ME) and hybridized with 1×10^7 cpm of nick-translated ^{32}P-labelled metallothionein cDNA (approx. 2×10^8 dpm/μg) at 55°C in 50% formamide, 3 $\times$ SSC. The membranes were washed (to 0.2 $\times$ SSC at 42°C), dried and exposed to Kodak X-Omat-5 film with intensifying screens.

3. RESULTS

We have shown that transfection of tk genes, but not other DNA sequences [10], into Ltk− aprt− cells leads to restoration of the capacity to

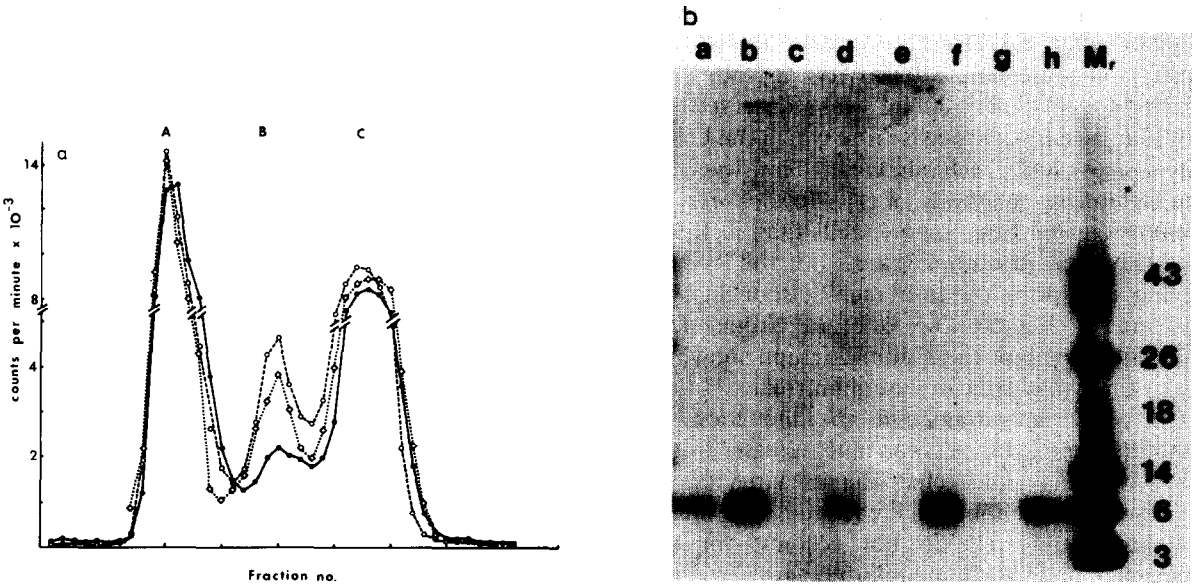

Fig.1. Induction of metallothionein synthesis by cadmium. (a) Sephadex G75 chromatography of extracts from Ltk− aprt− (●—●), C6 (○---○) and 81.7 (◇---◇) cells incubated with 15 μM cadmium for 4 h and labelled with [^{35}S]cysteine. Peak A contains the bulk of cellular protein, peak B metallothionein and peak C free cysteine. (b) Electrophoresis of metallothionein peaks. Pooled fractions (peak B) were concentrated and analysed by SDS-PAGE after reduction and alkylation. Samples were from L-929 (a,b), Ltk− aprt− (c,d), C6 (e,f) and 81.7 (g,h) cells treated without (a,c,e,g) or with 15 μM cadmium (b,d,f,h). M_r shows the position of M_r ($\times 10^{-3}$) standards.

respond to interferon in a substantial number of tk+ clones [9,13]. We observed that many tk+ clones (12 of 13 tested) were considerably more resistant to the toxic effects of cadmium sulphate than the Ltk− aprt− parent. Enhanced resistance to cadmium could be due to an increased constitutive level of transcription from the endogenous metallothionein genes or a potentiation of their capacity to be activated by exposure to heavy metals. Metallothionein synthesis was assayed in cultures exposed to cadmium sulphate (15 μM) for 4 h and then radiolabelled with [^{35}S]cysteine for 3 h. In untreated cells a metallothionein peak is barely detectable on Sephadex chromatography (not shown). After treatment with cadmium a distinct peak is observed (fig.1a, peak B). tk(+) clones, such as C6 and 81.7, exhibited considerably larger peaks corresponding to the metallothionein fraction than Ltk− aprt− cells after cadmium treatment (fig.1a) even though equivalent amounts of radioactivity were present in the total protein fraction (peak A). Fractions corresponding to the metallothionein peaks were pooled, lyophilized and aliquots corresponding to equal amounts of cellular protein analysed by SDS-PAGE after reduction and alkylation. An actinomycin D-sensitive increase in radioactivity migrating as metallothionein (M_r 7000) was observed in cadmium-treated L-929 cells from which the Ltk− aprt− cells had been derived. The level of metallothionein synthesis was 4-fold lower in cadmium-treated Ltk− aprt− cells than in L-929 cells. However, in both C6 and 81.7, the level of cysteine incorporation into metallothionein was considerably higher (3.5–3.9-fold from densitometric tracing of the autoradiograph shown in fig.1b) than that seen in cadmium-treated Ltk− aprt− cells, indicating that the induction of metallothionein synthesis was much greater in the cells containing transfected Herpes virus tk sequences. Cadmium-induced changes in cysteine precursor pool levels do not account for the enhanced labelling of metallothionein since the specific activity of the excluded fraction (peak A) was unaffected by cadmium treatment (ratio of induced to uninduced for Ltk− aprt−, C6 and 81.7 was 0.99, 1.03 and 1.02, respectively). Line C6 was derived from Ltk− aprt− by transfection with a plasmid bearing the HSV tk gene [9]. Clone 81.7 was obtained by transfecting Ltk− aprt− cells with plasmid pMK (containing the HSV tk coding sequence coupled to the mouse metallothionein promoter region) [14]. The increased inducibility of metallothionein synthesis is acquired independently of the means used to isolate the tk+ clones since C6 was selected in HAT-containing medium whereas 81.7 was derived by selecting with the neomycin analogue G-418 after co-transfecting plasmid pMK DNA with pAG60 containing the bacterial 'neo' gene [15]. In neither case was any selective pressure for cadmium-resistant mutants applied, ruling out the possibility that amplification of the endogenous metallothionein gene had taken place [11].

Quantitation of metallothionein synthesis in various cadmium-treated cell lines is shown in table 1. As seen above, L-929 cells synthesize 4–5-times more metallothionein than Ltk− aprt− cells. Enhancement in the level of metallothionein induction is observed not only in clones containing an intact HSV tk gene (C6 and 81.7) but also in cells containing fragments of this gene (68.6 and

Table 1

Induction of metallothionein synthesis by cadmium

	Cell-line	cpm	Relative synthesis
Expt A			
	Ltk− aprt−	2604	1.0
	C6	6580	2.5
	81.7	8225	3.2
	68-C6	9020	3.5
	92-D1	7758	3.0
	L-929	14513	5.6
Expt B			
	Ltk− aprt−	3481	1.0
	92-A2	3540	1.0
	92-A7	4121	1.2

Sub-confluent monolayers (10 cm dishes) were treated with cadmium sulphate (15 μM) and labelled with [^{35}S]cysteine as described in the legend to fig.1. A volume of each extract containing equivalent amounts of acid-insoluble radioactivity was made 0.5% in SDS, heated to 65°C for 10 min and subjected to chromatography on Sephadex G-75. Radioactivity in the metallothionein peak was summed. The incorporation relative to that of Ltk− aprt− is also presented

92-D1). These clones were obtained by transfecting a *Pst*I fragment (corresponding to the 5′-half of the tk gene) and a *Pst*I-*Pvu*II fragment (corresponding to the 3′-half of the gene) respectively into Ltk− aprt− cells together with plasmid pAG60. Clones were selected for neomycin resistance by growth in G-418. Both of these clones contain tk sequences and exhibit sensitivity to interferon as shown by resistance to vesicular stomatitis virus and by induction of oligo 2′,5′ A synthetase activity ([13]; J.A.L., in preparation). Thus, a functional tk gene or enzymatic activity is not required for restoration of interferon responsiveness or enhanced inducibility of metallothionein. The presence of tk-related sequences is required, however, since clones which received only the neomycin resistance gene (92-A2 and 92-A7) showed the same level of metallothionein synthesis as Ltk− aprt− cells after treatment with cadmium (table 1). These clones were also resistant to the anti-viral effects of interferon (not shown).

Metallothionein mRNA levels were quantitated by both dot-blot analysis of cytoplasmic extracts (cytoblots) and by Northern gel blotting of cytoplasmic RNA. As shown in fig.2, mRNA levels increase to a maximal value by 4–6 h after treatment with cadmium. Induction of mRNA was sensitive to actinomycin D (not shown) indicating that the response to cadmium is at the transcriptional level. Both the cytoblot and Northern gel procedures revealed a lower degree of induction of metallothionein mRNA in Ltk− aprt− cells

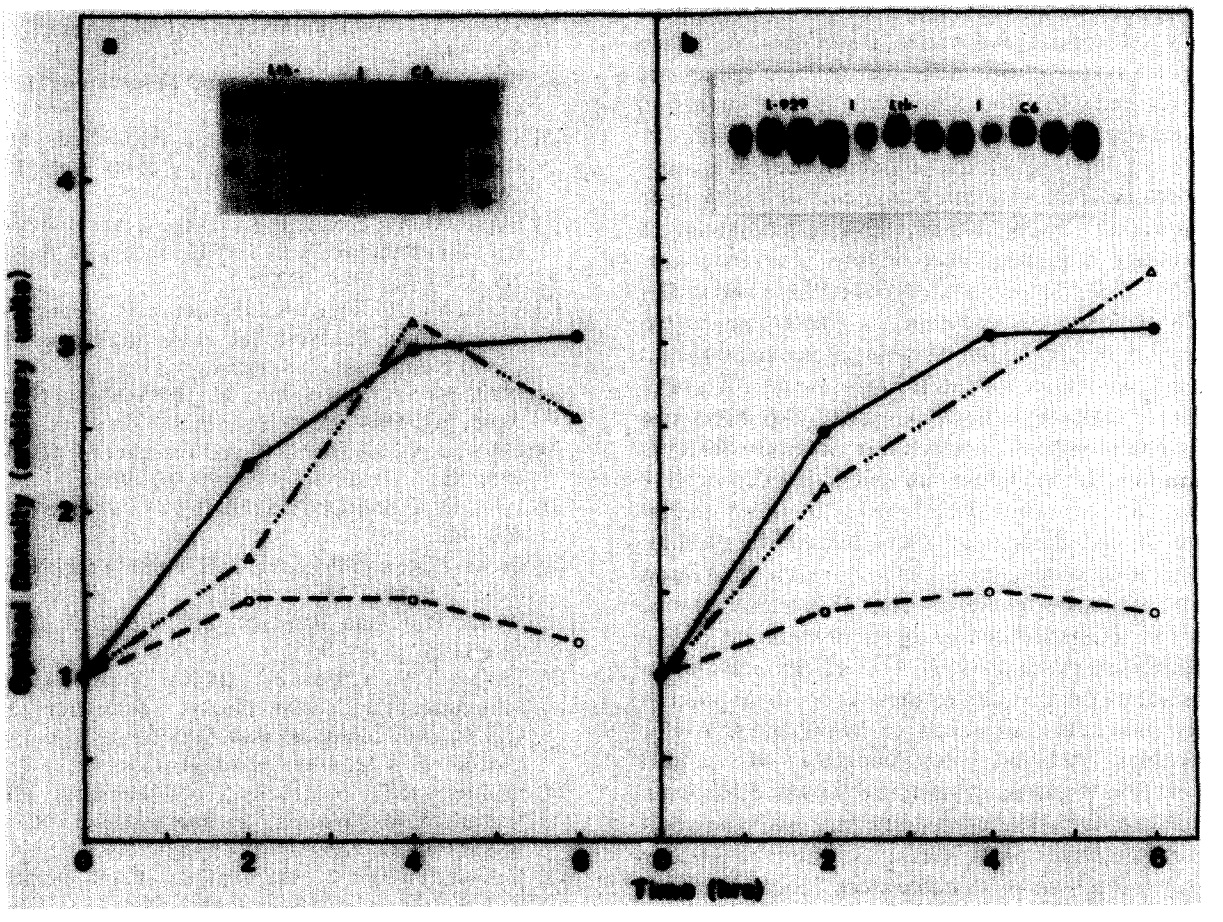

Fig.2. Induction of metallothionein mRNA by cadmium. RNA was extracted from cells treated with 0 or 15 μM cadmium sulphate for the times shown and analysed by dot-blotting (a) or by Northern blotting (b). Blots were quantitated by densitometry. Results for L-929 (●—●), Ltk− aprt− (○---○) and C6 (△-..-△) are shown.

(1.5-fold) than in L-929 cells. Clone C6 showed a level of induction similar to that of L-929 cells (3–4-fold).

4. DISCUSSION

Our previous studies have shown that Ltk− aprt− cells have lost the capacity to respond to interferon by activating an anti-viral state [9,10]. The reason for this unresponsiveness is unclear but the cells still possess cell-surface receptors as shown by the ability of interferon to inhibit cell growth and to induce certain interferon-responsive genes (J.A.L. and Shan, B., in preparation). The lack of thymidine kinase activity is not correlated with interferon resistance [10,16]. Interferon sensitivity can be restored in 50–100% of clonal isolates when an HSV [9] or chicken [13] tk gene is introduced. Such an effect is not observed when control transfections with aprt or 'neo' genes are performed [10] and sensitivity is lost in tk(−) revertants [9] showing that specific DNA sequences are involved. Furthermore, we have shown that sub-genomic fragments of the tk gene containing closely related (80% homologous) 28 base-pair sequences also restore interferon sensitivity [13]. The results described here show that HSV tk sequences also confer a greater capacity to activate the expression of endogenous metallothionein genes. Since metallothionein is one of several genes which are induced by exposure to interferon [5] it is tempting to relate our previous observations on interferon responsiveness of Ltk− aprt− cells and their tk+ derivatives with the potentiation of metallothionein inducibility. Metallothioneins and other genes responsive to interferon share upstream regulatory sites [17] which may be subject to the influence, direct or indirect, of the transfected tk sequences. This effect may be to potentiate the capacity of these genes to respond to their inducers (interferon or heavy metals). For unknown reasons, the capacity of certain interferon-response genes to be activated has been muted in the Ltk− aprt− line but, with the additional presence of tk elements, these regulatory systems become serviceable once more. The sensitivity of the well-defined metallothionein promoter to a tk sequence should facilitate analysis of the mechanism by which these tk sequences can affect a whole family of interferon-sensitive genes.

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