Volume 279, number 2, 310–312 FEBS 09432 February 1991

ADONIS 001457939100147S

# Thionein (apometallothionein) can modulate DNA binding and transcription activation by zinc finger containing factor Sp1

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Received 19 December 1990

A number of transcription factors contain so-called zinc finger domains for the interaction with their cognate DNA sequence. It has been shown that removal of the zinc ions complexed in these zinc fingers abrogates DNA binding and transcription activation. Therefore we wanted to test the hypothesis that the activity of transcription factors could be regulated by physiological chelators of zinc. A prominent candidate for such a chelator is the Cys-rich protein thionein (apometallothionein) that is inducible by heavy metal loads, and by other environmental stimuli. Here we show with DNA binding and in vitro transcription assays that thionein indeed can inactivate the zinc finger-containing Sp1 in a reversible manner. By contrast, transcription factor Oct-1, which binds DNA via a homeo-domain, i.e. a helix-turn-helix motif not involving zinc ions, is refractory to thionein action. We propose that modulation of intracellular thionein concentration is used for the coordinated regulation of a large subset of genes whose transcription depends on zinc finger proteins.

Metallothionein; Thionein; Zinc; Zinc finger protein; Sp1; Gene regulation

## 1. INTRODUCTION

Zinc finger proteins are one of the largest classes of DNA-binding proteins involved as transcription factors in controlling gene expression [1,2]. Their metal component bound to histidine and cysteine or cysteine residues alone is an indispensable cofactor for the function of these proteins. Thus, as shown for several transcription factors, removal of zinc by added chelating agents results in reversible loss of DNA-binding and transcriptional regulation in vitro [3–6]. This observation also suggests that modulation of the free zinc concentration by a physiological metal scavenger could provide a mechanism for regulating gene expression. An endogenous scavenger with a potential for such action is the Cys-rich protein thionein (apometallothionein). This ubiquitous 6 kDa protein has a very high affinity for zinc and related metal ions and has long been known to be responsible for their intracellular sequestration when they are supplied in excess [7]. Thionein is inducible by a variety of heavy metal ions, notably zinc, as well as by many regulators of cellular activity such as steroid hormones, interleukins, cAMP and diacylglycerol and is believed to play a major but as yet undefined role in the cellular response to chemical and physical stress, in tissue repair and in certain stages of embryonic development (or cellular differentiation) [7,8]. Sp1 is a transcription factor common to all vertebrates with a DNA binding domain at its C-terminus that contains three zinc fingers [9]. In the present study, we demonstrate in an in vitro system that by removing zinc, thionein can suppress binding of transcription factor Sp1 to DNA, resulting in modulation of gene transcription.

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## 2. MATERIALS AND METHODS

### 2.1. *Preparation of metallothionein and thionein*

Metallothionein was isolated from rabbit ($(Cd,Zn)_7$-thionein) and human liver ($Zn_7$-thionein) as described [10]. Thionein was prepared by acidification of native metallothionein and metal removal by gel filtration in 10 mM HCl [11].

### 2.2. *Preparation of nuclear extract and bandshift assay*

The preparation of Hela cell nuclear extract and bandshift assay were performed as described [12]. Before use, thionein was adjusted to pH 7.4 with 0.1 M Tris-HCl, pH 8.0. Prior to the addition of the oligonucleotide probe, the extract was preincubated with thionein at room temperature for 20 min; in the restoration experiment, the extract was incubated for 20 min with thionein, then zinc was added and the reaction was incubated for another 20 min.

### 2.3. *Construction of DNA templates and in vitro transcription*

The templates were as described before [12]. In vitro transcription with Hela cell nuclear extract was done as previously described except that thionein or $Zn_7$-thionein (where indicated) was incubated with nuclear extract for 20 min before the transcription reaction was initiated [6].


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## 3. RESULTS

### 3.1. *Thionein inhibits the binding of transcription factor Sp1 to DNA*

Previous bandshift and footprinting studies have shown that binding of Sp1 to DNA is abolished in the presence of the chelator EDTA or 1,10-phenanthroline. This effect was attributed to the loss of zinc from the zinc fingers [6]. We have now used the same assay system to study the effect of thionein on this zinc-dependent DNA/protein interaction. Hela cell nuclear extract was used as the source of Sp1. An Sp1 responsive DNA segment from the mouse MT gene promotor was used as a representative Sp1 binding site. Fig. 1 shows that incubation of the $^{32}$P-end labelled DNA fragment with nuclear extract resulted in a striking retardation of the label after electrophoresis through a native polyacrylamide gel (Fig. 1A, lanes 1 and 2). That this retardation is attributable to the specific binding of Sp1 is documented by competition with an excess of unlabelled Sp1 binding segment (Fig. 1A, lane 3).

To test if thionein interferes with binding of Sp1 to DNA, different amounts of thionein were added to the nuclear extract and incubated at room temperature for 20 min prior to mixing it with the DNA label. Fig. 1A (lane 7) illustrates that as little as 1.7 $\mu$M thionein almost completely abolish binding of Sp1 to the probe. A comparable inhibition of complex formation is observed in the presence of an about 200-fold higher concentration of EDTA (data not shown). No inhibition is observed when native $Zn_7$-thionein is included in the reaction mixture (Fig. 1A, lanes 11,12). As expected the inhibition of Sp1 binding to labelled DNA caused by thionein is reversed by the addition of zinc (Fig. 1B, lanes 4,5). The same results were obtained when instead of the mouse MT gene promoter an Sp1-responsive sequence from the Herpes simplex virus immediately early-3 (HSV 1E-3) gene promoter was used as a DNA probe (data not shown). By contrast, binding of the zinc-independent 'octamer' transcription factor (Oct-1, or OTF-1) to its DNA binding site was not affected by thionein (data not shown, see also [6]).

### 3.2. *Thionein abolishes the transcription activator function of Sp1*

To address the functional relevance of the inhibition of Sp1 binding by thionein we also investigated its effect on the activation of in vitro transcription by Sp1. As a template for transcription, we used an OVEC reporter gene with a duplicated strong Sp1 binding sequence (5'-GGGGCGGGGC-3') for Sp1 located 11 bp upstream of the TATA box [6,12]. As a control template we used OTF-OVEC with a heptamer and 'octamer' sequence (ATGCAAAT) instead of the Sp1 element [6]. The reference gene OVEC-REF containing a 186 bp fragment of the SV40 enhancer upstream of the TATA box was used as internal standard in each transcription assay [12]. All templates were transcribed by the Hela cell nuclear extract [12]. The RNA synthesized in the in vitro assay was monitored by S1-nuclease mapping [12]. As indicated in Fig. 2, the transcription from Sp1-OVEC template was reduced to a low

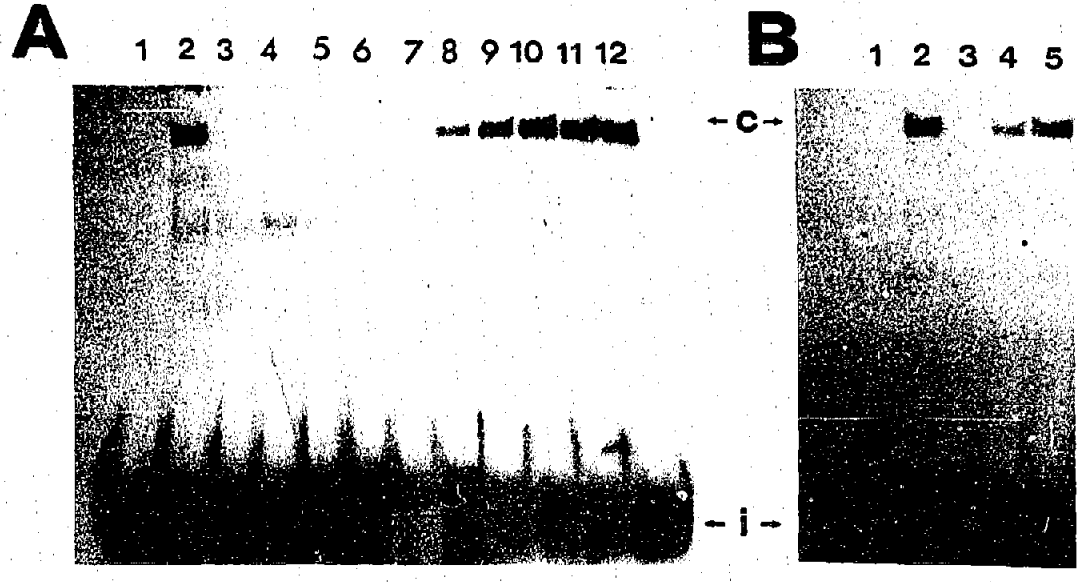


Fig. 1. Bandshift assay with Hela cell nuclear extract. (A) Thionein (apoMT) inhibition of binding of Sp1 to DNA. (Lane 1) No extract; (lane 2) with extract; (lane 3) competition with 1000-fold excess unlabelled Sp1 binding sequence; (lanes 4–10) addition of 13.6, 6.8, 3.4, 1.7, 0.85, 0.43, 0.22 $\mu$M thionein, respectively; (lanes 11,12) addition of 6.8 and 13.6 $\mu$M $Zn_7$-thionein, respectively. i, input fragment; c, major complex. (B) Restoration of Sp1 binding to DNA in the presence of thionein by addition of zinc. (Lane 1) No extract; (lane 2) with extract; (lane 3) addition of 11.7 $\mu$M thionein; (lanes 4, 5) addition of 11.7 $\mu$M thionein, and then 54, 100 $\mu$M zinc, respectively.

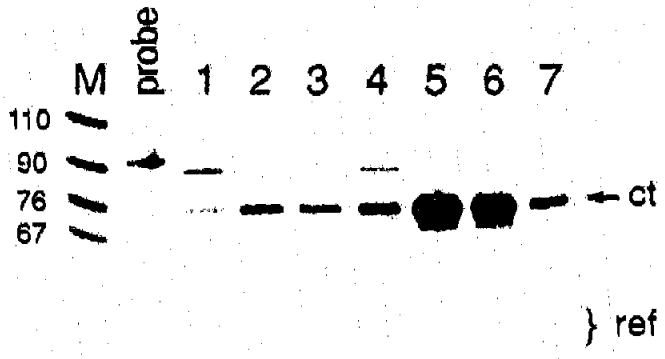


Fig. 2. In vitro transcription in a Hela cell nuclear extract and S1 nuclease mapping of RNA products. (Lane 1) TATA-only template; (lanes 2-4) OVEC-OTF template (lane 2, control; lane 3, with 50 $\mu$M $Zn_7$-thionein; lane 4, with 50 $\mu$M thionein); (lanes 5-7) OVEC-Sp1 template (lane 5, control; lane 6, with 50 $\mu$M $Zn_7$-thionein; (lane 7) with 50 $\mu$M thionein; S1 probe, $^{32}$P-end labelled 93 nucleotide probe extending between positions −18 and +75 on the non-coding strand of the $\beta$-globin gene. M, *Hap*II-digested pBR 322 marker DNA.

level by adding 50 $\mu$M thionein to the reaction (Fig. 2, compare lanes 5 and 7), whereas transcription from the template with the 'octamer' sequence (OTF-OVEC) was insensitive to the presence of thionein (Fig. 2, lane 4). Likewise, addition of native $Zn_7$-thionein instead of thionein to the transcription reaction had no inhibitory effect (Fig. 2, lanes 3 and 6). We thus conclude that thionein can be an efficient inhibitor of the zinc finger protein Sp1.

## 4. DISCUSSION

The mechanism by which thionein interferes with binding to DNA and transcriptional activity of the zinc finger protein Sp1 is most likely the sequestration of zinc from the free ion pool and from readily dissociable zinc complexes. Thionein avidly binds zinc and other heavy metal ions by forming unique oligonuclear metal/thiolate complexes. In the well-characterized $Zn_7$- and $Cd_7$-thionein, the metal ions (Me) and the 20 cysteine (Cys) side chains are partitioned into a $Me_3Cys_9$ and a $Me_4Cys_{11}$ cluster enfolded by the amino-terminal and carboxyl-terminal half of the polypeptide chain, respectively [7]. The apparent average association constant governing zinc complexation by thionein is strongly pH-dependent and is estimated to be of the order of $10^{14}$ $M^{-1}$ [11]. This is larger than the minimum value of $10^{12}$ $M^{-1}$ estimated for a representative zinc finger complex [13], and is also larger than the apparent association constant of the Zn-EDTA complex which depends both on pH and on the concentration of the competing magnesium ions in the assay mixture. Hence, the differences in the actual binding constants explain the present results. As a corollary, the magnitude of the association constant for zinc in $Zn_7$-thionein limits its capacity to transfer the metal to weaker acceptors. Thus, while there are several reports that native metallothioneins in vitro reactivate apometalloenzymes with higher affinity for zinc [14], it is thermodynamically not feasible to activate zinc-dependent proteins of low affinity with $Zn_7$-thionein. Differences in binding constant may also explain why in our experiments thionein had no inhibitory effect on the zinc-containing RNA polymerase II in the extract, as illustrated by the unimpaired transcription with an 'octamer' binding sequence in the promoter (Fig. 2, lane 4).

Our in vitro results suggest that changes in the intracellular supply of thionein will also affect zinc concentration in vivo. From mass law principles it is obvious that the ratio of the in vivo concentrations of $Zn_7$-thionein and thionein defines the equilibrium concentration of free zinc and that this zinc-donor/zinc-acceptor couple contributes importantly to the maintenance of cellular zinc homeostasis. Since, within cells, thionein is much less abundant than $Zn_7$-thionein, it follows, moreover, that even minor increments of thionein by increased biosynthesis afford substantial reduction in the concentration of free zinc and thereby reduce its availability to zinc-dependent systems. Thionein is inducible by a number of stimuli, such as steroid hormones, interleukins, interferon, cAMP, diacylglycerol and others. Thus we propose that modulation of intracellular thionein concentration is used for the coordinated regulation of a large subset of genes whose transcription depends on zinc finger proteins.

*Acknowledgements:* We are indebted to Fritz Ochsenbein for expert graphical work and to Dr Pamela Mitchell and Dr Hans P. Müller for critical reading of the manuscript and valuable comments. This work was supported by the Kanton of Zürich, and the Swiss National Science Foundation.

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