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Metal-dependent SV40 viruses containing inducible enhancers from the upstream region of metallothionein genes

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We have isolated SV40 recombinant viruses which are dependent on heavy metal ions for efficient propagation. They were obtained after-co-transfection of enhancerless SV40 DNA (the so-called enhancer trap) with sonicated DNA from the mouse metallothionein-I (mMT-I) or human metallothionein-IIA (hMT-IIA) upstream regions. To substitute for the SV40 enhancer, these viruses have incorporated a segment of the immediate upstream region of the metallothionein genes. Two recombinant viruses of the SVMT-I type carry segments of the mMT-I gene from positions −73 to −187 and −39 to −194 inverted with respect to their natural configuration. The overlapping segment contains two of the four metal-responsive elements involved in the induction of the mMT-I gene by heavy metal ions. The SVMT-II recombinant virus contains a segment of the hMT-IIA gene from position −39 to −366 which harbors the metal- and hormone-responsive elements of the hMT-IIA gene. Insertion of the mMT-I segment downstream of a rabbit β-globin test gene enhances β-globin transcription upon metal ion stimulation. This shows that the immediate upstream region of the mouse metalliothionein-I gene, when detached from its TATA box, can act as an inducible enhancer. It may be generally true that the enhancer/promoters of inducible genes are composed of several regulatory sequence elements which are interspersed with constitutive elements. The number and spatial arrangement of these elements probably determines the basal versus induced level of expression.

Key words: enhancer/metalliothionein/metal induction/SV40/transcription control

Introduction

The transcription of a number of eukaryotic genes can be switched on within a very short time upon the action of an environmental stimulus. Typical examples of such inducible genes are the heat shock and interferon genes. Another well-characterized family of inducible genes are the metallothionein genes.

Metallothioneins are small cysteine-rich proteins which play an important role, not only in the detoxification of heavy metal ions, but apparently also in heavy metal homeostasis in eukaryotic organisms (for review, see Hunziker and Kägi, 1985). Bivalent metal ions, in particular Zn^{2+}, Cd^{2+} and Cu^{2+}, are effective inducers of transcription of metallothionein genes. Functional analyses of cloned and mutated metallothionein genes have revealed several 'metal-responsive elements' (MREs) within 200 bp upstream of the transcription initiation (cap) site. The MREs are sequence motifs of ~15 bp which appear to be essential for the metal ion-dependent induction of metallothionein gene transcription (Carter *et al.*, 1984; Karin *et al.*, 1984; Stuart *et al.*, 1984; Searle *et al.*, 1985a). These MREs are presumably the binding sites of a transcription factor(s) which facilitates transcription by RNA polymerase II (Seguin *et al.*, 1984).

In addition to the metal ion-dependent inducibility, mammalian metallothionein genes are also inducible by steroid hormones (Hager and Palmiter, 1981; Karin *et al.*, 1984), interferon (Friedman *et al.*, 1984), a hitherto unidentified factor in the acute-phase response (Durnam *et al.*, 1984) and by other agents. For all of these inducers, specific responsive DNA segments seem to exist which differ in their position and sequence from the MREs (Durnam *et al.*, 1984; Friedman *et al.*, 1984; Karin *et al.*, 1984). Therefore, the induction of metallothionein gene transcription by various environmental stimuli seems to be a complex process regulated by the interaction of several target DNA sequences, the corresponding transcription factors and RNA polymerase II.

We wanted to find out whether the regulatory upstream regions of metallothionein genes can act as transcriptional enhancers. Enhancers were originally described in viruses as long range activators of transcription (Banerji *et al.*, 1981; Moreau *et al.*, 1981). Subsequently, enhancers were shown to be important for the expression of cellular genes, such as for immunoglobulin genes (Banerji *et al.*, 1983; Gillies *et al.*, 1983; Neuberger, 1983; Picard and Schaffner, 1984; Queen and Stafford, 1984; for review of cellular enhancers, see Gluzman, 1985). Enhancers differ from proximal promoter elements like the TATA box in that they strongly activate correct transcription of a linked gene over long distances of >1000 bp, and even from a position downstream of the cap site (for reviews, see Gluzman, 1985; Serfling *et al.*, 1985).

For the isolation of enhancer elements from mouse metallothionein-I and human metallothionein-IIA DNA, we used the so-called enhancer trap assay. The enhancer trap is an enhancerless and therefore non-infectious linear SV40 molecule which is co-transfected with putative enhancer DNA into monkey CV-1 cells. Intracellular ligation-repair processes create recombinant DNA molecules, and viable viruses which have integrated a heterologous enhancer are selected (see Weber *et al.*, 1984). The assay has been successfully applied to the isolation of enhancers from a number of viruses (Boshart *et al.*, 1985; Dorsch-Häsler *et al.*, 1985; Schirm *et al.*, 1985; Weber and Schaffner, 1985).

Here we show that the assay is also applicable to the isolation of enhancers from cellular genes. In two independent enhancer trap exeriments we were able to isolate metal ion-inducible SV40 viruses which, in place of the SV40 enhancer, have incorporated overlapping segments of the upstream region of the mouse metallothionein-I (MT-I) gene. One of these upstream segments contains all the MREs, the other contains only the two distal MREs. In a further experiment, we also isolated a recombinant virus containing part of the upstream region of the human MT-IIA gene (hMT-IIA). This fragment not only harbors the MREs, but also

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the steroid hormone-responsive element of the hMT-IIA gene (Karin *et al.*, 1984).

By cloning the mouse MT-I enhancer element downstream of a rabbit β-globin gene and by testing its activity in transient expression assays, we could show that the immediate upstream region of the mouse MT-I gene can act as an inducible enhancer. This constitutes the first demonstration of an inducible enhancer in the strict sense, since previous work with inducible controlling elements either did not address the question of remote control including activation from a downstream position (Pelham and Bienz, 1982; Chandler *et al.*, 1983; Karin *et al.*, 1984; Fujita *et al.*, 1985; Ryals *et al.*, 1985), or activation from a downstream position was not shown for a heterologous gene (Goodbourn *et al.*, 1985). In metallothionein genes, the enhancer/promoter region apparently is composed of both regulatory and constitutive elements, a situation which may be representative of many regulated genes.

Results

Construction and structure of metal ion-dependent SV40 viruses

For the enhancer trap experiments, DNA of clones pXMT and pHS1 containing the upstream sequences of the mouse mMT-I gene and the human hMT-IIA gene, respectively, was sonicated and co-transfected with enhancer trap DNA into monkey CV-1 cells (see Materials and methods). Virus growth was observed in three independent experiments. The analysis of two recombinant viruses, SVMT-I-1 and SVMT-I-2 (for details see Materials and methods), showed that DNA segments of 115 and 156 bp, respectively, from the upstream region of the mMT-I gene had been incorporated into the virus genome inverted relative to its

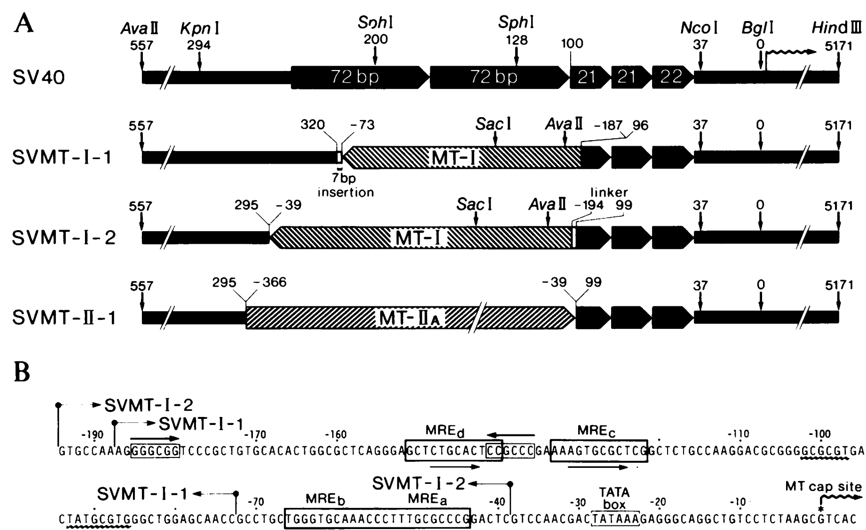

Fig. 1. Structure of the enhancer/promoter region of metal ion-dependent SV40 recombinant viruses. **(A)** Schematic maps of enhancer/promoter regions in SV40, SVMT-I-1, SVMT-I-2 and SVMT-II-1. Solid bars indicate SV40 DNA, hatched bars metallothionein DNA, open bars linker DNA or inserted extra bases. The arrowheads of the SV40 72-bp enhancer repeats and of the promoter 21/22-bp repeats indicate the direction of early transcription, the wavy arrow near 0 (= origin of replication) designates the main start site of early transcription. The arrowheads of MT segments indicate the orientation of mMT-I and hMT-IIA DNA within the SVMT-I and SVMT-II viruses, respectively. **(B)** Sequence of the enhancer/promoter segment of mouse metallothionein-I gene. Both SVMT-I viruses were sequenced from their *Hind*III site at position 5171 to the *Ava*II site of late SV40 DNA at position 557. The boundaries of the MT-I enhancer DNA incorporated into the viruses are indicated. The MREs a – d are boxed and underlined by arrows according to Stuart *et al.* (1984). Two additional boxes, with arrows above them, represent two inverted GGGCGG motifs, and the wavy lines indicate two short stretches of alternating purine/pyrimidine. Note the two base differences to the mMT-I sequence originally published by Glanville *et al.*, (1981): the insertion of an A residue at position −112 and the deletion of a C residue at position −157.

Table I. Growth properties and T antigen expression of recombinant SVMT-I and SVMT-II viruses

Viruses (cloned viral DNA)	Growth properties[a] ~50% of cells lysed (days)		T antigen fluorescence[b] (in % of cells)		Ratio of induction
	−	+	− (No. Exp.)	+ (No. Exp.)	
SVMT-I-1	>23[c]	19	0.035 (2)	0.37 (2)	10.4
SVMT-I-2	16/>23[c]	16/19.5	0.24 (4)	2.85 (4)	11.9
SVMT-II-1	<40[c]	25	n.d.	n.d.	n.d.
pBSV3x (SV40 wild-type)	10/10.5	10/10.5	2.31 (4)	2.0 (4)	0.9
pET-1 (enhancerless SV40)	n.d.	n.d.	0 (2)	0 (2)	n.d.

[a]A 10 μl aliquot from the 5 ml lysate of CV-1 cells infected with the respective clone was frozen and thawed three times and dissolved in 1 ml DMEM. The medium was split and pipetted into two 50 mm Petri dishes with CV-1 cells seeded 1 day before. After 45 min incubation at 37°C with occasional tilting, fresh DMEM medium was added. For metal ion induction (+) 10^{-4} M Zn^{2+} and 0.5 × 10^{-6} M Cd^{2+} were added. One day later the medium was changed and the ion concentration was increased to 2 × 10^{-4} M Zn^{2+} and 10^{-6} M Cd^{2+}. −, control dishes.
[b]2 μg DNA were transfected into CV-1 cells using the DEAE-dextran transfection protocol. One half of the transfection mixture was used for the metal ion-induced dish (+), the other one for the non-induced control dish (−) (see Materials and methods). Cells of five to seven areas of 0.145 mm each were counted, and the numbers were used for the extrapolation of percentage of stained cells as described in Banerji *et al.* (1981). The numbers of transfection experiments are given in brackets.
[c]No sign of infection on day when experiment was terminated.

natural orientation in front of the mMT-I gene. In the third virus, SVMT-II-1, a segment of 328 bp from the upstream region of the hMT-IIA gene was incorporated in its natural orientation.

SVMT-I-1 contains the mMT-I sequences from nucleotide −73 to −187. At position −187 the mMT-I segment was directly fused to nucleotide 96 of the SV40 early promoter, whereas between positions −73 of mMT-DNA and 320 of SV40 late DNA, seven extra nucleotides were inserted (see Figure 1). SVMT-I-2 harbors a similar mMT-I DNA segment spanning nucleotides −39 to −194 integrated between the *Kpn*I and *Xba*I sites of the enhancer trap, respectively (Figure 1).

The mMT-I DNA sequences incorporated into SVMT-I-1 and SVMT-I-2 are shown in Figure 1B. The segment incorporated in SVMT-I-2 harbors all of the MREs thought to be responsible for the heavy metal ion-dependent induction of the mouse MT-I gene (Carter *et al.*, 1984; Stuart *et al.*, 1984; Searle *et al.*, 1985a, 1985b). In SVMT-I-1 only the two distal MREs are present whereas the two most proximal MREs forming an inverted repeat are lacking.

The hMT-IIA segment in SVMT-II-1 spans nucleotides −39 to −366 and therefore comprises both the multiple MREs and the hormone-responsive element(s) of the hMT-IIA gene (Karin *et al.*, 1984; see Figure 1A). Studies on the binding of rat liver glucocorticoid receptor to hMT-IIA promoter sequences revealed a strong receptor binding site between nucleotide positions −240 and −270 and an additional weak binding site centered around nucleotide −324 (Karin *et al.*, 1984). Both binding sites are part of the hMT-IIA segment in our SVMT-II-1 virus.

The SV40-MT recombinant viruses were dependent on heavy metal for efficient propagation, but in some cases the dependence was not absolute (Tables I and II). This latter finding remains to be further analyzed since, by contrast, no SV40-MT recombinants were recorded in several enhancer trap experiments performed in the absence of heavy metal (data not shown). As shown in Figure 3, the metal treatment of cells transfected with recombinant pSVMT-I DNA results in the appearance of brilliantly fluorescent nuclei, similar to the immunofluorescence of cells transfected with wild-type SV40. On the other hand, only a low number of dull fluorescent nuclei was observed in the absence of heavy metal ions.

The propagation of the SVMT-II-1 virus is even more dependent on metal ions than that of the SVMT-I viruses (Table I). By contrast, SVMT-II growth was unaffected by dexamethasone treatment (our unpublished results). This is probably due to the lack or underrepresentation of hormone receptors in CV-1 cells (M. Karin, personal communication).

The activity of MT-DNA can be influenced by SV40 enhancer and early promoter sequences

In additional enhancer trap experiments we wanted to test whether the presence of further SV40 enhancer or SV40 early promoter sequences linked to the mMT-I segment would stimulate the overall activity of MT sequences without abolishing the induction by metal ions. To this end, the *Bgl*I-*Hinf*I fragment from the enhancer region of pSVMT-I-1o (see Materials and methods) was co-transfected together with the 5 kb long *Sph*I-*Bgl*I fragment of wild-type SV40 into CV-1 cells. The *Sph*I-*Bgl*I fragment of SV40 comprises part of the SV40 enhancer including 50 bp of one of the two 72-bp repeats and all enhancer DNA sequences from the 'late side' of SV40. Figure 2A depicts the structure of the enhancer/promoter region of one of the resulting viruses, SVMT-I-121. Its mMT-I segment has been fused between positions −66 and −69 to the SV40 late DNA between positions 204 and 207. SVMT-I-121 and all viruses of this type showed a high level of T antigen expression, vigorous growth and almost no inducibility by heavy metals (see Table II). This indicates that the SV40 enhancer overrides the metal responsiveness of the mMT-I DNA segment.

In the course of constructing MT-SV40 recombinants, we also obtained a virus with a duplicated SV40 early promoter, designated SVMT-I-17. Somewhat unexpectedly, metal inducibility was even more pronounced in recombinant SVMT-I-17 than in recombinants with a single early promoter. SVMT-I-17 virus was obtained when the *Bgl*I-*Hinf*I fragments of the SVMT-I-1o enhancer/promoter region was co-transfected with enhancer trap DNA into CV-1 cells. The sequence of its enhancer/promoter region is presented in Figure 2B (see also Materials and methods). Due to the duplication of 87 bp of its early promoter,

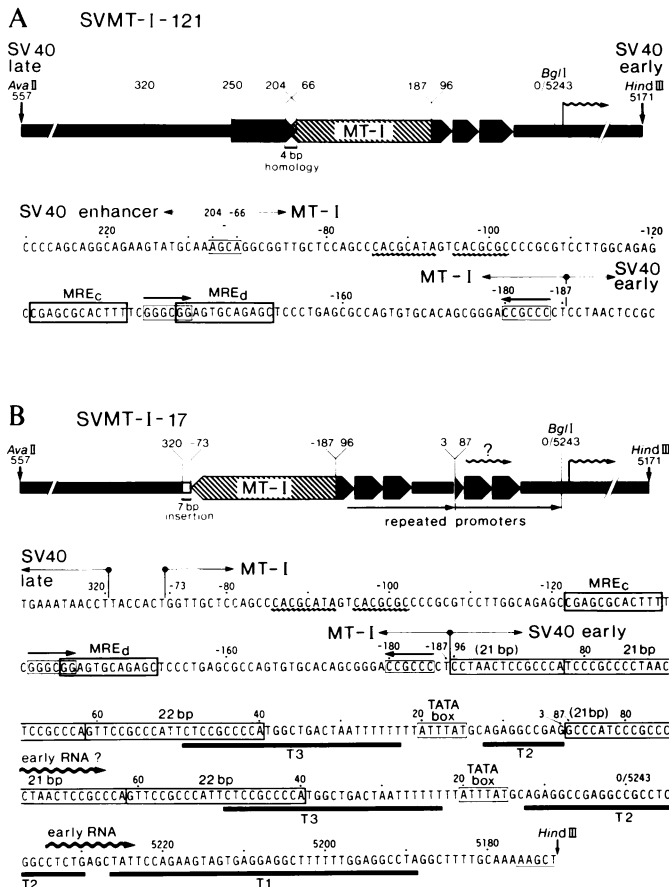

Fig. 2. Structure of the enhancer/promoter region of recombinant viruses SVMT-I-121 and SVMT-I-17. For the symbols see Figure 1. **(A)** SVMT-I-121 was generated by the fusion of a *Bgl*I-*Hinf*I fragment of SVMT-I-1o (see Materials and methods) and a *Sph*I-*Bgl*I fragment of SV40 wild-type DNA co-transfected into CV-1 cells. The non-matching *Hinf*I-*Sph*I ends apparently were joined by homologous recombination involving an identical 4-bp sequence between nucleotides −66 to −69 of the mMT-I sequence and nucleotides 204−207 of the SV40 DNA (similar recombination events involving 5-bp and 6-bp homologies have been observed in previous enhancer trap experiments: Weber *et al.*, 1984; Boshart *et al.*, 1985). **(B)** SVMT-I-17 was generated by the fusion of *Bgl*-*Hinf*I fragment of SVMT-I-1o (see Materials and methods) and an (enhancerless) SV40 enhancer trap molecule co-transfected into CV-1 cells. On one side, the cellular ligation/repair processes yielded seven additional nucleotides between position −73 of mMT-I DNA and 320 of SV40 late DNA. At the other side, despite an extended homology, the molecules were joined by end-to-end fusion resulting in the duplication of the SV40 early promoter. T1, T2, T3, T antigen binding site. (The absence of the dominant T antigen binding sites, T1 and T2, in the early promoter next to the MT-I segment could be responsible for the high metal inducibility of T antigen expression in SVMT-I-17; see Discussion).

Table II. The influence of an early promoter duplication, and of additional SV40 enhancer DNA on growth properties and T antigen fluorescence of viruses SVMT-I-17 and SVMT-I-121, respectively

Virsues (cloned viral DNA)	Growth properties ~50% of cells lysed (days)		T antigen fluorescence (in % of cells)		Ratio of induction
	−	+	− (No. Exp.)	+ (No. Exp.)	
SVMT-I-17	>30[a]	17/17/16/16	0.03 (3)	1.78 (3)	59
SVMT-I-1o	n.d.	n.d.	0.04 (1)	0.5 (1)	12.5
SVMT-I-11	17	16	0.07 (1)	0.52 (1)	7.4
SVMT-I-12	17	16	0.05 (1)	0.35 (1)	7
SVMT-I-19	23/23	19/19	0.08 (1)	0.7 (1)	8.6
SVMT-I-112	20/21	18/18	0.15 (1)	0.52 (1)	3.5
SVMT-I-121	10/13	10/9	2.3 (2)	2.55 (2)	1.1
SVMT-I-122	10/11.5/11.5	9.5/10/10	1.8 (1)	2.3 (1)	1.3

For the determination of growth properties and T antigen fluorescence see Table I. The structures of enhancer/promoter region of SVMT-I-17 and SVMT-I-121 are shown in Figure 2. SVMT-I-11, SVMT-I-12, SVMT-I-19 and SVMT-I-112 are derived from SVMT-I-10 (see Materials and methods). Their structure is similar to that of SVMT-I-1 (Figure 1) but this was not confirmed by sequencing. SVMT-I-17 contains a duplicated SV40 early promoter. SVMT-I-122 carries a substantial part of the SV40 enhancer very similar to SVMT-I-121.
[a]No sign of infection on day when experiment was terminated.

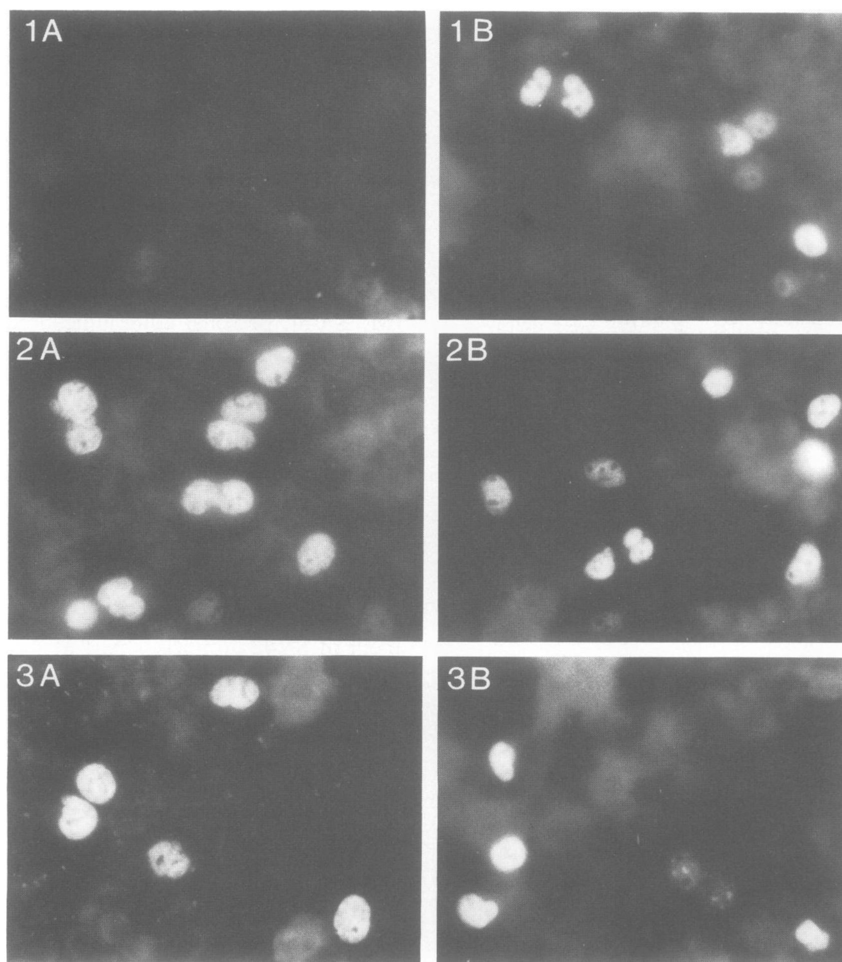

Fig. 3. Metal ion-dependent induction of T antigen expression by the MT-I enhancer. The recombinant constructs pSVMT-I-17 **(1)** and pSVMT-I-121 **(2)** as well as pBSV3x **(3)**, a clone with three SV40 wild-type genomes, were transfected in parallel into CV-1 cells using the DEAE-transfection protocol (see Materials and methods). One hour after the DMSO shock, metal ions were added to one of two duplicate Petri dishes (No. 1B, 2B and 3B). Dishes 1A, 2A, 3A were used as non-induced controls. Fourty hours later, cells were fixed and stained for nuclear T antigen by indirect immunofluorescence (see Materials and methods).

SVMT-I-17 has a second early TATA box and probably a second functional start site for early transcription, ~90 bp upstream of the natural SV40 start sites (Figure 2B). T antigen expression of pSVMT-I-17 was found to be extremely metal ion-inducible (see Table II). The early promoter duplication of pSVMT-I-17 is responsible for its high T antigen induction, because T an-

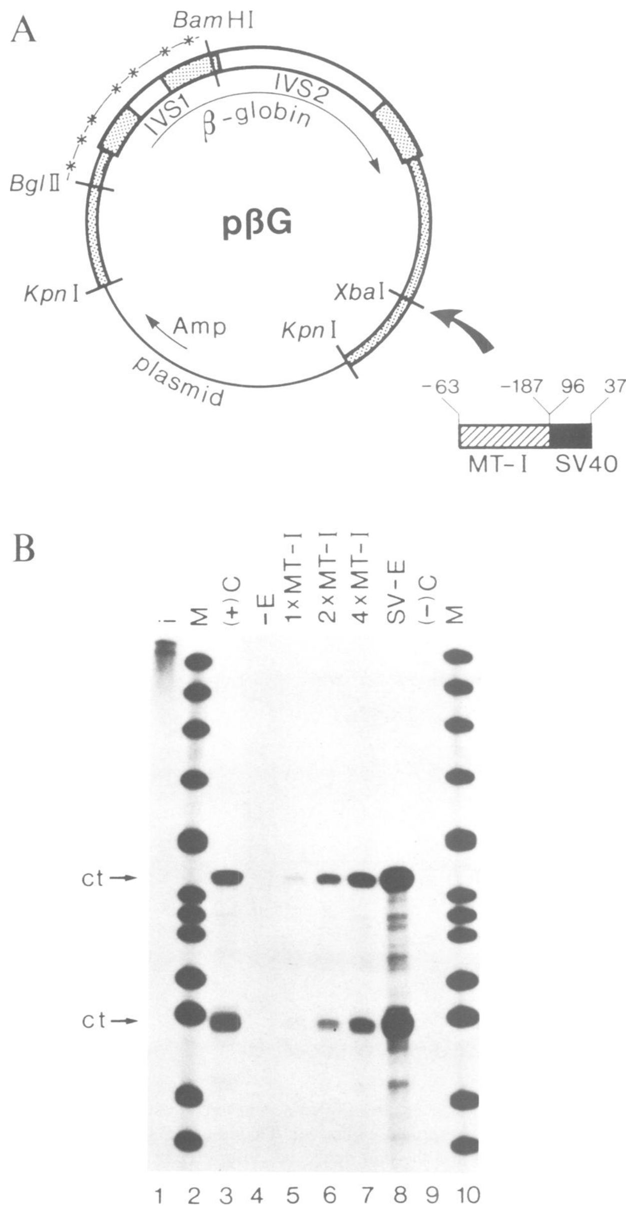

Fig. 4. Induction of β-globin transcription directed by the mouse metallothionein I-enhancer. **(A)** Map of clone pβG containing a 4.7-kb fragment of rabbit β-globin gene. By introducing one, two or four copies of mMT-I enhancer DNA (the *NcoI-HinfI* fragment of pSVMT-I-1o; see Materials and methods) into the *XbaI* site of pβG the clones pβGMT-1x, pβGMT-2x and pβGMT-4x were obtained. As a positive control a clone with a 200-bp SV40 enhancer DNA fragment in the *XbaI* site of pβG (see Materials and methods) was used. For SP6 mapping the *BglII-BamHI* fragment (★ ★ ★) was cloned in the plasmid pSP6-5, labelled anti-strand RNA probes were synthesized by the SP6 polymerase system (Melton *et al.*, 1984) and used for the mapping of globin mRNA as shown in **(B)**. **(B)** SP6-RNA mapping of RNA from HeLa cells transfected with MT-enhancer containing clones. The clones pβGMT-1x, pβGMT-2x and pβGMT-4x **(lanes 5–7)**, pβG **(lane 4)** and pβG+SV40 enhancer **(lane 8)** were transfected into HeLa cells using the calcium phosphate protocol (see Materials and methods). One hour after the DMSO shock, metal ions were added, and 36 h after metal induction cytoplasmic RNA was isolated and mapped with *BglII-BamHI* fragment anti-strand RNA. In **lane 1** (i) full-length input RNA was added. The protected RNA fragments of hybridization reactions between the SP6 probe and rabbit globin mRNA (positive control) or yeast tRNA (negative control) are shown in **lanes 3** and **9**, respectively. M, *HpaII*-digested pBR322 marker DNA **(lanes 2 and 10)**. ct, correctly initiated transcripts.

tigen inducibility of an otherwise identical recombinant with a single early promoter (SVMT-I-1) was several fold lower than that of SVMT-I-17. Therefore, duplication of the SV40 early promoter, which itself does not seem to contain metal ion-responsive elements, potentiates inducibility by the mMT-I segment (see Tables I and II and Discussion).

The MT-I promoter DNA acts as an inducible enhancer element

To address the question of whether the mMT-I DNA segment, like a *bona fide* enhancer, is able to stimulate transcription of a test gene from a downstream position, we cloned the mMT-I segment from nucleotide −63 to −187 including some adjacent SV40 early promoter sequences into the *XbaI* site of the plasmid pβG (see Materials and methods). pβG harbors the rabbit β-globin gene with a single *XbaI* site located ~3 kb downstream of its cap site and ~1.5 kb downstream of its poly(A) addition site (Figure 4B). The three resulting plasmids, pβGMT-1x, pβGMT-2x and pβGMT-4x, containing one, two or four copies of the mMT-I segment, were transfected into HeLa cells. After incubation of the cells for 40 h in the presence of metal ions, cytoplasmic RNA was extracted and analysed for the presence of correct globin transcripts by SP6 mapping (Melton *et al.*, 1984). With a genomic globin SP6-clone (see Figure 4B), two specific fragments of 205 nucleotides and 145 nucleotides were generated. These fragments are indistinguishable from those of genuine rabbit β-globin mRNA indicating correct rabbit globin transcription in the transfected cells (Figure 4, lane 3). Moreover, an increasing copy number of the mMT-I segment potentiates globin gene expression. While a single mMT-I segment enhances transcription only weakly, the presence of two and four mMT-I copies resulted in a strong stimulation of transcription by heavy metal ions (see Figure 4, lanes 5–7).

To demonstrate the inducibility of the mMT-enhancer, each construct was used to transfect two plates containing HeLa cells. One of them was incubated in the presence of heavy metal ions, the other was used as the non-induced control. In some experiments a reference gene, REFΔ (see Figure 5B and Materials and methods), was also included as an internal transfection control. The cytoplasmic RNA of both plates was analysed in parallel for globin transcripts by quantitative S1 nuclease mapping (see Figure 5A).

These metal ion-induction experiments reveal a strong β-globin specific band with cytoplasmic RNA of induced HeLa cells transfected with clone pβGMT-4x. Quantitation by Cerenkov counting of excised β-globin bands (from lanes 2 and 3 in Figure 5C, designated ct) demonstrated a 16-fold induction of β-globin transcripts by metal ions. By contrast, the activity of the SV40 enhancer is at most marginally (1.4-fold) increased by metal ions. In comparison with the globin transcription directed by the SV40 enhancer, the mMT-I enhancer-containing construct pβGMT-4x yielded about one fifth of the level of β-globin transcripts in metal ion-induced HeLa cells (see Figure 5C and 5D and also Figure 4).

The 21-bp repeats of SV40 early promoter are unable to act from a downstream position

The mMT-I enhancer segment used in the above-mentioned experiments also contains 60 bp of DNA from the SV40 early promoter with five potential binding sites for transcription factor Sp1 (see Dynan and Tjian, 1985). Since our construct pβGMT-4x harbors 20 of these motifs, we wanted to determine if they contribute significantly to the observed enhancer effect. To this end we cloned one or several (up to eight) copies of the SV40 early promoter segment, from position 37 to 100, downstream of the

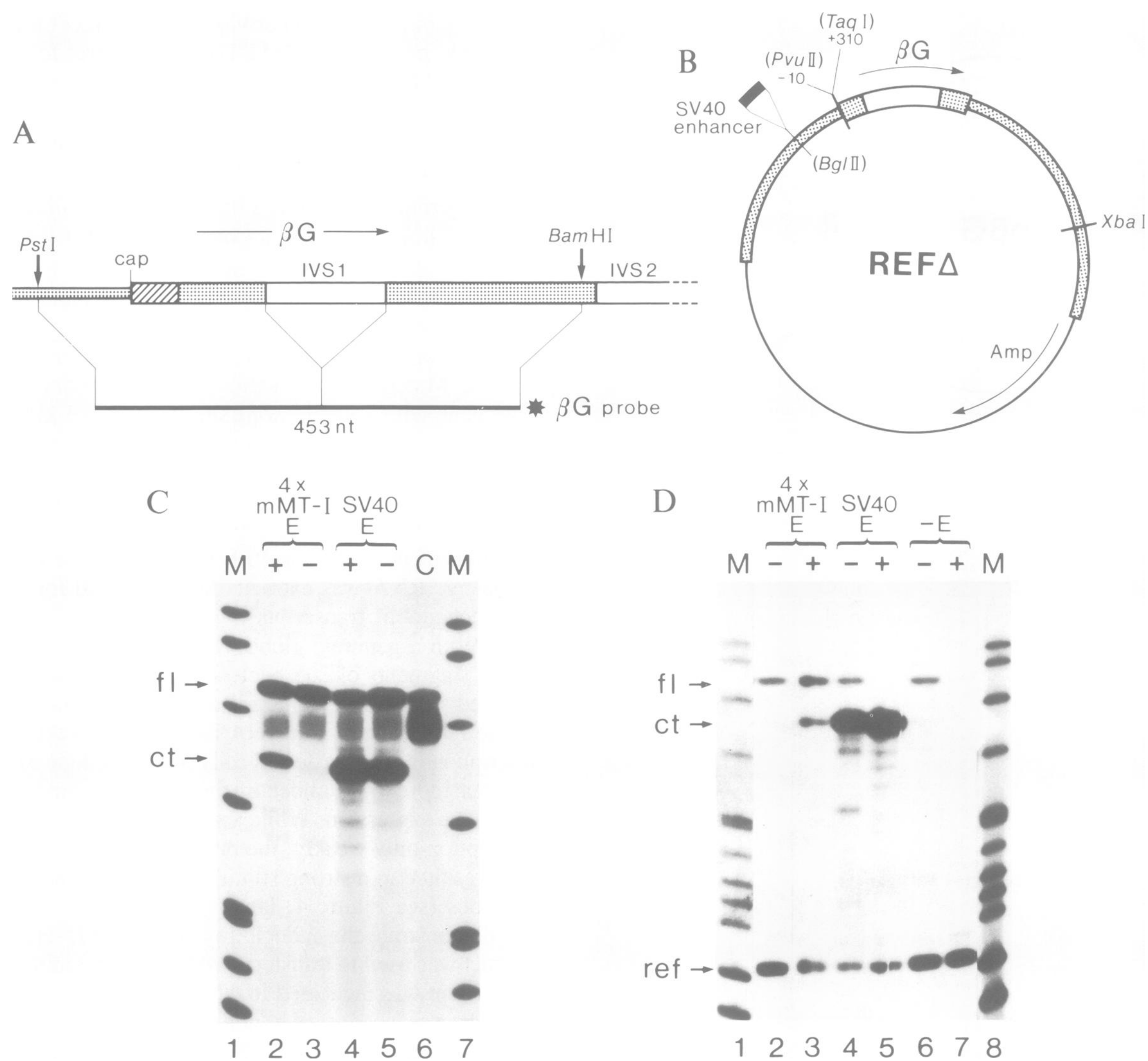

Fig. 5. Metal ion-induced transcription of the β-globin gene directed by the mouse metallothionein I-enhancer. **(A)** β-globin gene probe used for the S1 nuclease mapping. The probe is derived from a clone lacking the first intervening sequence (IVS1) as described earlier (de Villiers and Schaffner, 1983). The hatched bar corresponds to the 5′-untranslated mRNA segment, the thick dotted bar to exon segments and the thin dotted bar to upstream sequences of the globin gene. The single-stranded DNA probe of 453 nucleotides (filled bar below) was end-labelled as indicated by the terminal asterisk. **(B)** Map of REFΔ, the internal globin reference clone. The rabbit β-globin gene was modified by deleting the segment spanning positions −10 to +310 (see Picard and Schaffner, 1985). **(C)** S1 nuclease mapping of RNA from metal ion-induced and non-induced HeLa cells. The cells were transfected with the mMT-I enhancer clone pβGMT-4x **(lanes 2 and 3)** and the SV40 enhancer containing clone pβG+SV40E **(lanes 4 and 5)**. +, RNA from metal ion-induced HeLa cells; −, RNA from non-induced cells. **Lane 6** (c) shows the hybridization between yeast tRNA and S1 probe. ct, signal for correctly initated transcripts. fl, full length input DNA probe. **(D)** S1 nuclease mapping of RNA from metal ion-induced and non-induced HeLa cells co-transfected with the reference plasmid REFΔ and the MT-I enhancer clone pβGMT-4x **(lanes 4** and **5)** or the enhancerless clone pβG **(lanes 6** and **7)**, respectively. Each of the clones was mixed with REFΔ DNA at a 10:1 mass ratio. ref, signal for the RNA of REFΔ DNA.

β-globin gene (clone pβG; see Materials and methods). SP6 mapping of RNA from transfected HeLa cells revealed, however, no measurable enhancement of β-globin transcription (results not shown). This indicates that these Sp1 binding sites alone are unable to stimulate β-globin gene transcription from a downstream position.

Discussion

The enhancer trap and the construction of metal ion-inducible viruses

The enhancer trap approach (Weber *et al.*, 1984) has previously been used to isolate enhancers from a variety of viruses, such as from human and mouse cytomegalovirus (Boshart *et al.*, 1985; Dorsch-Häsler *et al.*, 1985), Herpes saimiri (Schirm *et al.*, 1985) and the long terminal repeat of Rous sarcoma virus (Weber and Schaffner, 1985). Until now, however, we have been unable to isolate enhancers from cellular genes by this technique. The cellular enhancers isolated so far were found to be associated with highly active cell type-specific genes, as exemplified by the enhancers of immunoglobulin genes (Banerji *et al.*, 1983; Gillies *et al.*, 1983; Neuberger, 1983; Picard and Schaffner, 1984; Queen and Stafford, 1984). Immunoglobulin enhancers are only active in lymphoid cells but not in CV-1 cells, the host cells of SV40. Enhancer trap experiments with total mammalian cellular DNA (Weber *et al.*, 1984) and with cloned housekeeping genes have also been negative (our unpublished data). Housekeeping genes are generally transcribed at a low rate and may therefore contain no, or only weak enhancers, which seem to be unable to replace the strong SV40 enhancer.

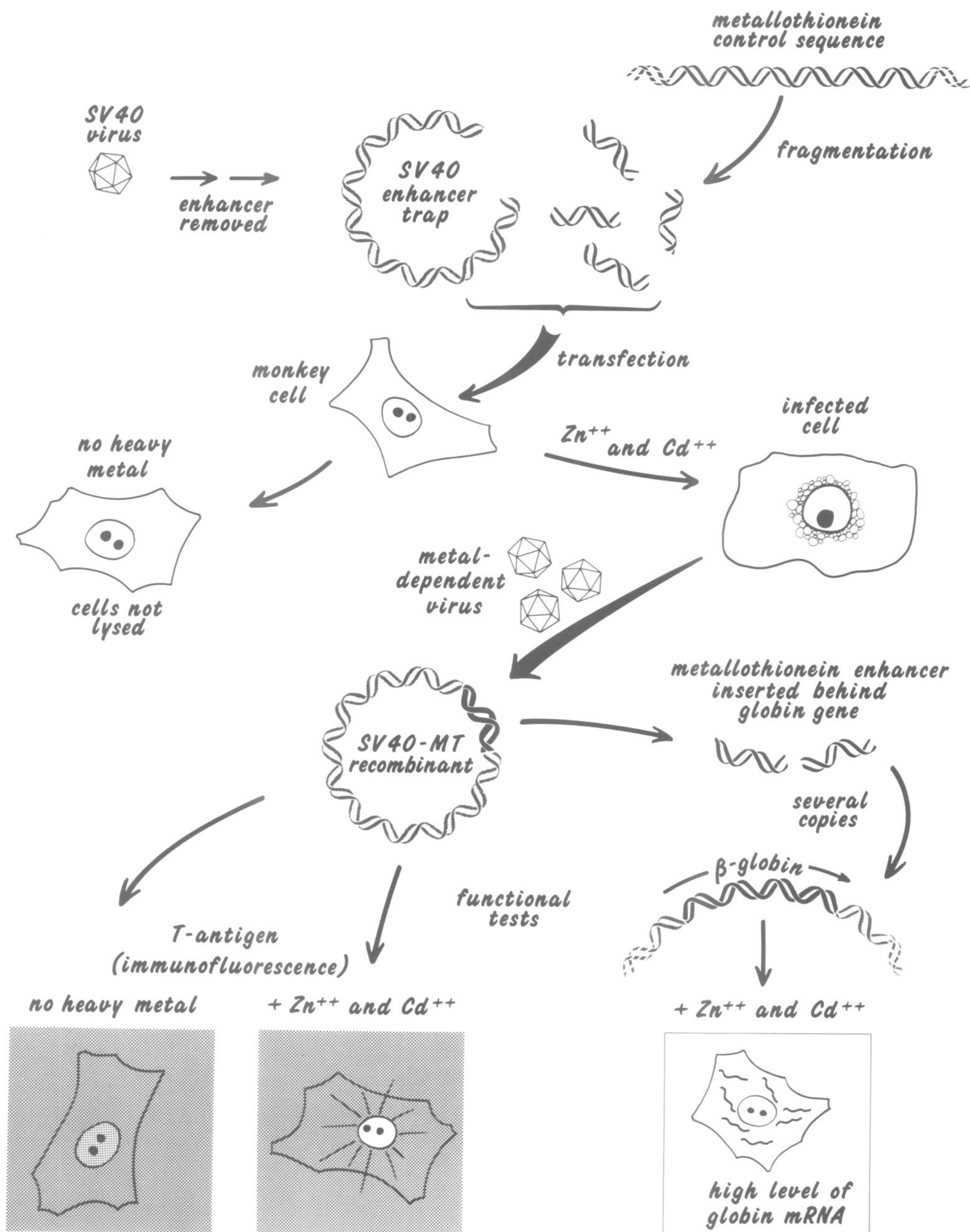

Fig. 6. Protocol for the selection and analysis of heavy metal-inducible SV40 viruses. For details see text and Materials and methods.

The metallothionein genes were good candidates for the isolation of inducible enhancers since these genes are strongly transcribed in the presence of metal ions and since their expression is not strictly cell type-specific. Enhancer trap experiments performed in the presence of metal ions indeed yielded recombinant SV40 viruses containing the inducible upstream sequences of MT genes in place of the deleted SV40 enhancer (for a summary of protocols see Figure 6).

For the mouse MT-I gene we recovered two viruses containing overlapping segments of the immediate upstream region of the mMT-I gene (see Figure 1). These segments harbor MREs and also 'constitutive' sequence motifs which probably determine the basal level of mMT-I gene expression (see below). The incorporated mMT-I segments lack not only the TATA box but also the far upstream sequence elements involved in mMT-I gene induction in the acute-phase response (Durnam *et al.*, 1984) and by interferon (Friedman and Stark, 1985).

We have also isolated an enhancer element from the human MT-IIA gene comprising the upstream sequences from positions −39 to −366. This segment is about twice the size of the mMT-I enhancer and carries, apart from the MREs, the hormone-responsive element of the hMT-IIA gene around position −260 and an additional hormone receptor binding site around position −324 (Karin *et al.*, 1984). Since recombinant SVMT-II-1 was isolated in the absence of hormones it is likely that the far upstream sequences from the hMT-IIA gene contribute to the basal and/or metal-induced enhancer activity.

Inducible and constitutive sequence motifs within the mouse MT-I enhancer

The immediate upstream region of the mouse MT-I gene can act as an inducible transcription enhancer. We have shown here that

this DNA segment is able to stimulate gene expression when located upstream of the SV40 early transcription unit as well as when located ~3 kb downstream from the β-globin cap site. The mMT enhancer element functions efficiently only in the presence of heavy metal ions. This shows that MREs described as metal ion-inducible promoter elements of MT genes (Carter *et al.*, 1984; Karin *et al.*, 1984; Stuart *et al.*, 1984) play an important role in establishing the enhancer activity. Functional tests have shown that the consensus sequence TGCRCNC constitutes the core element of MREs of the mouse MT-I gene (Searle *et al.*, 1985b). There are three potential MREs of this type located around nucleotide positions −170, −145 and −125 in the mMT-I segment (position −63 to −187) which was used for testing the enhancer activity downstream of the β-globin gene. Two of them, called MREc (around −125) and MREd (−145), were shown to be inducible promoter elements when linked to a thymidine kinase test gene (Searle *et al.*, 1985b). In SVMT-I-2 both proximal elements, MREa and MREb, have also been incorporated (see Figure 1B). Somewhat unexpectedly, SVMT-I-2 shows an elevated basal level of T antigen expression as compared with SVMT-I-1, but the ratio of induced versus uninduced expression is about the same for both viruses (Table I). Therefore, the extra 40 bp in SVMT-I-2, which also include the (inverted and now promoter-distal) MREs a and b, do not potentiate inducibility but rather contribute to the basal level of expression.

In addition to the MREs there are several further sequence motifs within the mMT-I enhancer which may contribute to the stimulation of transcription. These include several potential binding sites for the transcription factor Sp1 and two short stretches of alternating purine/pyrimidine (Figure 1). Two of the putative four Sp1 factor binding sites which have the 'core' sequence GGGCGG and resemble the 'ideal consensus sequence' $^{G}_{T}$GGGCGG$^{GGC}_{AAT}$ (Dynan and Tjian, 1985), are located around nucleotide positions −138 and −183 in an inverted orientation (see Figure 1B). Around positions −90 and −100 there are two short stretches of alternating purine/pyrimidine which are probably involved in enhancing gene expression (Karin *et al.*, 1984; Hamada *et al.*, 1984; Herr and Gluzman, 1985).

The effect of linked SV40 regulatory DNA sequences on the activity of the mMT-I enhancer

We were interested to see whether the mMT-I enhancer in the non-induced state was perhaps acting as a negative control element capable of blocking the activity of a 'constitutive' enhancer. However, it became obvious that SV40 enhancer sequences linked to the mMT-I enhancer are able to override the metal inducibility of the MT-enhancer. This is shown by the T antigen expression and growth properties of viruses of the SVMT-I-121 type. Although these viruses harbor a complete mMT-I enhancer segment between the SV40 early promoter and part of the SV40 enhancer, they display properties similar to SV40 wild-type virus, i.e., high T antigen expression and vigorous growth in the presence as well as in the absence of metal ions (see Table II and Figure 3). Similarly, it was found by others that in acinar pancreas cells of transgenic mice the elastase enhancer is able to eliminate the inducibility of mMT-I upstream sequences (R. Palmiter, personal communication). It may, therefore, be generally true that highly active enhancers override the action of weaker enhancers.

In SVMT-I-17, the duplication of the SV40 early promoter leads to a significantly higher T antigen expression. Since enhancers act normally on the most proximal of tandem promoters (de Villiers *et al.*, 1983; Wasylyk *et al.*, 1983), early transcription in SVMT-I-17 probably starts downstream of the TATA box of the promoter next to the mMT-I DNA. This promoter segment lacks both strong T antigen binding sites T1 and T2 and only retains the low affinity site T3 (see Figure 2B). Therefore, the high T antigen inducibility in SVMT-I-17 might be due to the defective autoregulation of T antigen transcription. This hypothesis is amenable to experimentation by studying the metal ion-induced T antigen expression in recombinant viruses with mutated T antigen binding sites.

The enhancers of inducible eukaryotic genes

The metal-inducible enhancers in the immediate upstream region of metallothionein genes include sequences which, according to previous assays, are typical 'promoter' components. These upstream elements in their entirety can, therefore, be viewed depending on their context, as either an upstream promoter region or an enhancer.

Sequence analysis of a variety of enhancers has revealed the presence of a multitude of short conserved sequence elements (Banerji *et al.*, 1983; Lusky *et al.*, 1983; Hearing and Shenk, 1983; Weiher *et al.*, 1983; Boshart *et al.*, 1985; Dorsch-Häsler *et al.*, 1985; Herr and Gluzman, 1985). From these and other data we conclude that enhancers generally have a modular structure with highly redundant information in which conserved motifs of several kinds are separated by less conserved DNA sequences (Serfling *et al.*, 1985). These enhancer-promoter elements apparently bind cellular transcription factors, either of a constitutive type (such as the Sp1 factor binding to the GGGCGG motif) or of a regulatory type (such as a steroid hormone receptor, or the putative factor for metal induction of metallothionein genes). We have speculated that upon induction a regulatory factor, perhaps after an allosteric conformation change, forms a bridge between constitutive factors (Serfling *et al.*, 1985). The number and spatial arrangement of these DNA motifs presumably determines the basal versus induced level of transcription; e.g., as shown in Figure 4, the number of enhancer elements can be directly correlated to the transcription level. As suggested by the multiple regulation of metallothionein genes, eukaryotes seem to realize a great variety of regulatory networks by modular combinations of a set of DNA elements.

Materials and methods

Cell growth and DNA transfections

Human HeLa cells and green monkey CV-1 cells were grown in DMEM. The medium was supplemented with 2.5% fetal calf serum, 2.5% calf serum, 100 U/ml penicillin and 100 U/ml streptomycin. The calcium phosphate transfection protocol was that of Graham and van der Eb (1973) and of Wigler *et al.* (1978) with the modifications described in Weber *et al.* (1984). DEAE-dextran transfections were done according to de Villiers and Schaffner (1983) except that after transfection the cells were incubated for 4 min with 25% v/v dimethylsulfoxide (DMSO) in TBS buffer (25 mM Tris-HCl, 137 mM NaCl, 5 mM KCl, 0.7 mM CaCl, 0.5 mM $MgCl_2$, 0.6 mM Na_2HPO_4, pH 7.4). The cells were then washed twice with TBS and incubated with fresh medium. In all metal ion-induction experiments both Zn^{2+} ($1-2 \times 10^{-4}$ M Zn sulfate) and Cd^{2+} ($0.5-1 \times 10^{-6}$ M Cd acetate) were used. One hour after the DMSO shock of cells, 10^{-4} M Zn^{2+} and 0.5×10^{-6} M Cd^{2+} were added and the cells were incubated for 8 h. Then, the medium was changed and 2×10^{-4} M Zn^{2+} and 10^{-6} M Cd^{2+} were added.

Enhancer trap experiments and construction of recombinant viruses

In typical enhancer trap experiments, 1 μg of *Xba*I- and *Kpn*I-digested enhancer trap DNA (clone pET-1: Weber *et al.*, 1984) was mixed with 10 μg of sonicated DNA of the recombinant plasmid clones pXMT or pHS1, respectively. Clone pXMT (a gift of R. Gerard and Y. Gluzman) contains the upstream region of mouse MT-I gene from nucleotide position +62 to −600 in front of an SV40 T antigen gene; clone pHS1 (a gift of M. Karin) harbors the upstream region

of human MT-IIA gene from positions +70 to −780 which was cloned between the *Hin*dIII and *Bam*HI site in plasmid pUC8. Multiple vigorous sonication (ultrasonics W-10) was performed to obtain a main DNA fraction of ~300 bp in size.

Viral DNA was extracted from infected CV-1 cells by a modified alkaline procedure for the extraction of bacterial plasmid DNA (G. Magnusson, personal communication; see also Maniatis *et al.*, 1982). In short, the infected and partially lysed CV-1 cells were lysed to completion on Petri dishes for 2 min by 0.5% SDS/0.1 N NaOH, potassium acetate (pH 4.8) was added, and the material was transferred into Eppendorf tubes. After two phenol/chloroform deproteinization steps, the nucleic acids were precipitated by ethanol. The viral DNAs were linearized by *Bam*HI digestion and cloned into the *Bam*HI site of pUC8. The DNA of the enhancer/promoter region was sequenced after isolation and subsequent *Ava*II digestion of the viral *Hin*dIII-B/C fragments according to Maxam and Gilbert (1980).

In the first enhancer trap experiment using clone pXMT the recombinant virus SVMT-I-1o was isolated. SVMT-I-1o contains the mMT-I sequences from nucleotide position −63 to −187, and, in addition, a stretch of 60-bp DNA of unknown origin incorporated between nucleotide −63 of mMT-I DNA and position 420 of SV40 late DNA. Due to a *Hin*fI site near the 5′ terminus of the 60-bp DNA segment, this extra DNA was easily removed in further transfection experiments. To this end, *Bgl*I-*Hin*fI fragments from the enhancer/promoter region of SVMT-I-1o were isolated and co-transfected with pET-1 DNA into CV-1 cells. All viruses of the SVMT-I-11 to -112 type (see Table II) (including SVMT-I-17) were constructed in this way. SVMT-I-1 was generated after transfection of plasmid pSVMT-I-1 which was constructed by ligation of the corresponding *Sac*I-*Sal*I fragment of pSVMT-I-17 with that of pSVMT-I-121 [see Figure 2; the unique *Sac*I site is part of the mMT-I segment (see Figure 1A), the *Sal*I site is within pUC8)]. Viruses MT-I-2 and MT-II-1 were directly cloned from enhancer trap experiments.

Recombinant DNAs

All recombinant clones were constructed according to standard recombinant DNA techniques (Maniatis *et al.*, 1982). For the cnstruction of pβGMT-1x, -2x and -4x clones, the *Nco*I-*Hin*fI fragent of pSVMT-I-1 was isolated, subcloned in a pUC8-derived plasmid, excised and polymerized by T4 ligase. The ligation products were fractionated on a 1.8% low melting agarose gel, and the DNA of bands corresponding to 2−4 ligated segments was introduced into the *Xba*I site of pβG (see Figure 4A). In a similar way, the *Nco*I-*Xba*I fragment of pET-1 containing the major part of the SV40 early promoter was introduced as one or up to eight copies into pβG.

Immunofluorescence assay and RNA analyses

For immunofluorescence analysis, transfections were scaled down to 50 mm plates. Fixation of the cells and staining by T antigen-specific antibodies was performed as described (de Villiers and Schaffner, 1983).

Cytoplasmic RNA was isolated 40−44 h after transfection as described by de Villiers and Schaffner (1983). RNA mapping experiments with the SP6 polymerase system were essentially done as described by Melton *et al.* (1984) except that only 8 μg/ml RNase A and 60 U/ml RNase T1 were used. S1 nuclease mapping was performed with the single-stranded DNA probe shown in Figure 5A as described (de Villiers and Schaffner, 1983). Autoradiographs were quantitated by counting the Cerenkov radiation of excised bands.

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Using a different assay system, A.Haslinger and M.Karin (*Proc. Natl. Acad. Sci. USA*, **82**, in press) have found that a DNA segment upstream of the human MT-IIA gene has considerable enhancer activity even in absence of heavy metal ions. Meanwhile, we have further analyzed the MT enhancer segment present in SVMT-II-1 virus and find it in HeLa cells to be inducible by both heavy metals and glucocorticoid hormone (M.Pettersson and W.Schaffner, unpublished).