example, Selsing *et al.*[8] and Arnott *et al.*[9] have shown from model building that a helix bend is expected at the junction of B and A DNA regions.

In the case of K-DNA, we propose that oligo dA tracts have an altered helix structure, perhaps like the heteronomous (H) DNA structure devised by Arnott *et al.*[39] to explain the fibre diffraction data for poly(dA)·poly(dT). When these tracts are about 5 bp (half a turn) long, the junction bends will point in the same direction, yielding a net molecular bend, as shown in Fig. 3*b*. (Junction bends spaced by a full helical turn yield only a helix axis dislocation, not a net bend.) If these 5-bp tracts are separated by 5-bp spacers, then the bending will be additive and in the same plane. For example, with a junction bend of about 12° for the H–B DNA junction, half the angle suggested by Arnott *et al.*[7] for the A–B junction, then the projection of the DNA helix on the plane around the bending locus of K-DNA can be drawn as shown in Fig. 3*c*. Eight nearly inphase 12° bends generate an overall bend of about 90°, with the centre of bending located around bp 140, in close agreement with the results determined by the gel method for the sub-K fragment. This model readily explains the effect of distamycin on K-DNA, since it is known that the drug prefers the B helical form[38]. Reversion of dA tracts to B helix would eliminate junction bends; the same phenomenon may explain the disappearance of electrophoretic anomalies at elevated temperature[41]. Work is under way in our laboratory to explore quantitative application of this model to the K-DNA structure[42].

By analogy with the model for K-DNA bending, we propose that CAP may bend promoter DNA by induction of a structure such as the H form, creating junction bends. This proposal offers several possibilities for an influence of CAP binding on RNA polymerase–promoter interactions. For example, polymerase may recognize the H conformation directly, sliding from there to its normal interaction sites at −35 and −10. Alternatively, the H conformation may potentiate transient conversion of the adjacent promoter B-DNA to a conformation such as A which more readily binds polymerase. Finally, it is also possible that the role of DNA bending is primarily to create CAP–polymerase interactions which would not be sterically possible in a linear promoter. Further work will be required to sort through these possibilities.

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Characterization of DNA sequences through which cadmium and glucocorticoid hormones induce human metallothionein-IIA gene

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Deletion experiments have defined two stretches of DNA (genetic elements), lying close to the promoter for a human gene for metallothionein, that separately mediate the induction of the gene by heavy metal ions, particularly cadmium, and by glucocorticoid hormones. The element responsible for induction by cadmium is duplicated, yet a single copy is fully functional; the element responsible for induction by glucocorticoid hormones is coincident with the DNA-binding site for the glucocorticoid hormone receptor.

METALLOTHIONEIN (MT) genes, encoding low molecular weight heavy-metal binding proteins, are a useful experimental system for studying transcriptional regulation of higher eukaryotic genes. In mammals, MT genes are induced in a variety of cell lines and tissues by administration of either heavy metal ions or glucocorticoid hormones[1–4]. Both inducers are primary inducers of MT mRNA[5], acting at the transcriptional level[4,6]. The kinetic analysis of MT mRNA induction in HeLa cells, and the differential induction response to metal ions and glucocorticoids after inhibition of protein synthesis, suggest that the two classes of inducers act by independent mechanisms[5,7,8].

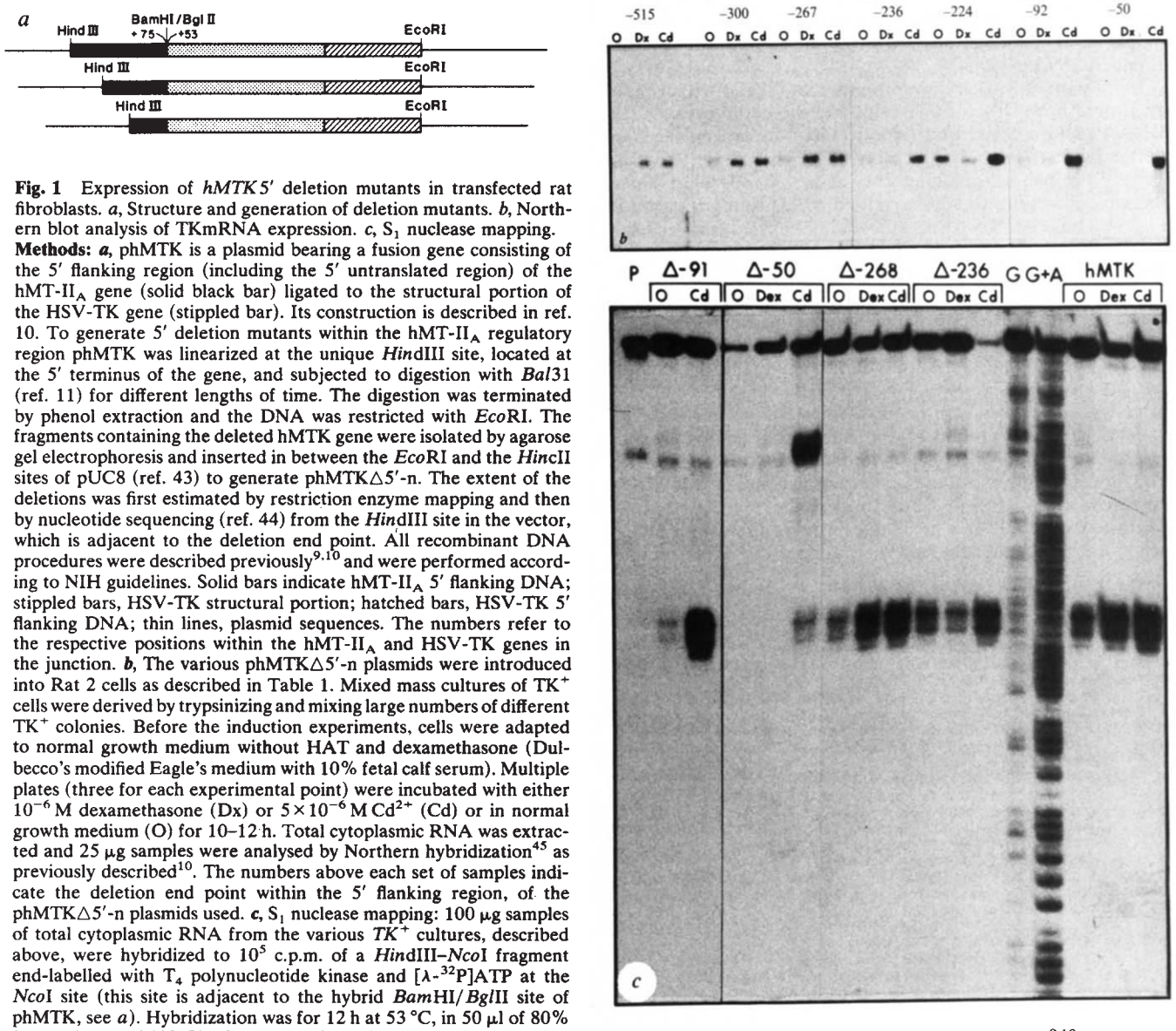

Fig. 1 Expression of *hMTK5′* deletion mutants in transfected rat fibroblasts. *a*, Structure and generation of deletion mutants. *b*, Northern blot analysis of TKmRNA expression. *c*, S_1 nuclease mapping. **Methods:** *a*, phMTK is a plasmid bearing a fusion gene consisting of the 5′ flanking region (including the 5′ untranslated region) of the hMT-II_A gene (solid black bar) ligated to the structural portion of the HSV-TK gene (stippled bar). Its construction is described in ref. 10. To generate 5′ deletion mutants within the hMT-II_A regulatory region phMTK was linearized at the unique *Hin*dIII site, located at the 5′ terminus of the gene, and subjected to digestion with *Bal*31 (ref. 11) for different lengths of time. The digestion was terminated by phenol extraction and the DNA was restricted with *Eco*RI. The fragments containing the deleted hMTK gene were isolated by agarose gel electrophoresis and inserted in between the *Eco*RI and the *Hin*cII sites of pUC8 (ref. 43) to generate phMTKΔ5′-n. The extent of the deletions was first estimated by restriction enzyme mapping and then by nucleotide sequencing (ref. 44) from the *Hin*dIII site in the vector, which is adjacent to the deletion end point. All recombinant DNA procedures were described previously[9,10] and were performed according to NIH guidelines. Solid bars indicate hMT-II_A 5′ flanking DNA; stippled bars, HSV-TK structural portion; hatched bars, HSV-TK 5′ flanking DNA; thin lines, plasmid sequences. The numbers refer to the respective positions within the hMT-II_A and HSV-TK genes in the junction. *b*, The various phMTKΔ5′-n plasmids were introduced into Rat 2 cells as described in Table 1. Mixed mass cultures of TK^+ cells were derived by trypsinizing and mixing large numbers of different TK^+ colonies. Before the induction experiments, cells were adapted to normal growth medium without HAT and dexamethasone (Dulbecco's modified Eagle's medium with 10% fetal calf serum). Multiple plates (three for each experimental point) were incubated with either 10^{-6} M dexamethasone (Dx) or 5×10^{-6} M Cd^{2+} (Cd) or in normal growth medium (O) for 10–12 h. Total cytoplasmic RNA was extracted and 25 μg samples were analysed by Northern hybridization[45] as previously described[10]. The numbers above each set of samples indicate the deletion end point within the 5′ flanking region, of the phMTKΔ5′-n plasmids used. *c*, S_1 nuclease mapping: 100 μg samples of total cytoplasmic RNA from the various TK^+ cultures, described above, were hybridized to 10^5 c.p.m. of a *Hin*dIII–*Nco*I fragment end-labelled with T_4 polynucleotide kinase and [λ-^{32}P]ATP at the *Nco*I site (this site is adjacent to the hybrid *Bam*HI/*Bgl*II site of phMTK, see *a*). Hybridization was for 12 h at 53 °C, in 50 μl of 80% formamide, 0.4 M NaCl, 40 mM PIPES *p*H 6.4, 1 mM EDTA. S_1 nuclease resistant DNA fragments were generated as described[9,10] and were displayed on a 10% denaturing acrylamide gel, together with the chemical degradation[44] products of the probe. The numbers above the lanes indicate the deletion end point. G and G+A indicate the products of the two purine-specific sequencing reactions[44]. P indicates the S_1 nuclease resistant products generated by hybridization of the probe to 100 μg of yeast tRNA. hMTK indicates S_1-resistant fragments formed by hybridization to RNA from cultures transfected with the intact hMTK gene as previously described[10]. RNA was derived from control cultures (O), dexamethasone-treated cultures (dex) or Cd^{2+} treated cultures (Cd). (The upper band present in the Cd-induced sample from the -50 deletion, is an artefact, caused by retarded migration of a portion of that sample due to incomplete removal of the RNA before loading.)

We recently introduced the cloned human MT-II_A gene (h*MT-II*$_A$) (ref. 9) into Rat 2 fibroblasts and found that it retains its responsiveness to both glucocorticoids and heavy metals. Also, a fusion gene (h*MTK*), constructed by joining the 5′ flanking region of hMT-II_A gene to the structural portion of the herpes simplex virus (HSV) thymidine kinase (*TK*) gene, is responsive to both inducers. The induction of both the intact hMT-II_A gene and of the hMTK fusion gene is due to increased transcription following either Cd^{2+} or dexamethasone administration[10].

To map the regulatory DNA elements and determine whether they serve as binding sites for various regulatory proteins we constructed progressive deletion mutants in the 5′ flanking region of the hMTK gene and tested them for both hormonal and heavy metal induction *in vivo*, after transfection into rat fibroblasts, and for their ability to bind to the rat liver glucocorticoid hormone-receptor complex *in vitro*.

We find at least three separate regulatory elements within the 5′ flanking region of the hMT-II_A gene, one, present in two copies, is involved in heavy metal ion induction. The second element centred around nucleotides −70 to −90 is important for the maintenance of the basal level of expression. The third element, centred 250–260 base pairs (bp) upstream to the start site of the transcription, is involved in hormonal induction. This element is also the binding site of the purified glucocorticoid hormone-receptor complex to the human gene.

Two upstream regulatory elements

We constructed a chimaeric gene consisting of 800 bp of the 5′ flanking region, containing the promoter elements, the cap site and the 5′ untranslated region of the hMT-II_A, fused to the transcribed portion of the HSV *TK* gene. When this fusion gene (h*MTK*) is transcribed from the hMT-II_A promoter it generates a functional TK mRNA at levels increased by treatment of transfected cells with either dexamethasone or Cd^{2+}. Both inducers act by increasing the transcription rate of the transfected gene[10]. We used the hMTK fusion for the construction of deletion mutants to characterize the regulatory elements of the hMT-II_A promoter. To generate a series of 5′ deletions, phMTK was linearized at the unique *Hin*dIII site, at its 5′ terminus, and

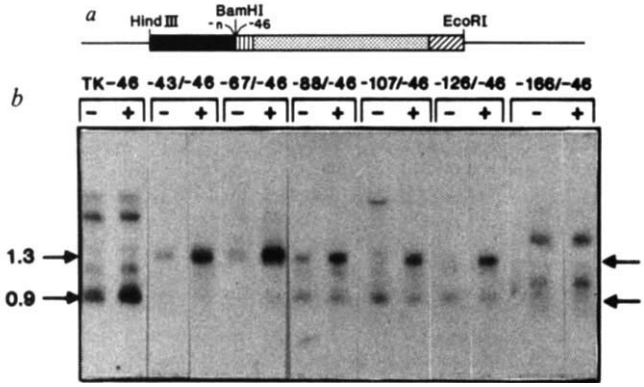

Fig. 2 Analysis of 3′ deletion mutants. *a*, Construction of 3′ deletions and hybrid promoters. The diagram illustrates the structure of the phMK-n/-46 fusions: solid bar, hMT-II$_A$ 5′ flanking DNA; stippled bar. HSV-TK transcribed region; hatched bars, HSV-TK 5′ and 3′ flanking DNA; thin lines, plasmid DNA. -n1, deletion end point within the hMT-II$_A$ gene; -46, deletion end point within TK gene. *b*, Expression of the phMT-n/-46 fusions. Arrows refer to 1.3 and 0.9-kb transcripts of HSV-TK gene, plus and minus signs denote presence or absence of Cd^{2+} before RNA extraction (see below).

Methods: *a*, To generate 3′ deletions in the hMT-II$_A$ gene 5′ flanking regions, p84H, a *Hin*dIII subclone of hMT-II$_A$ gene[9], was linearized at the single *Nco*I site (at the AUG initiator codon of the gene). The linearized DNA was digested for different times with exonuclease *Bal*31 (New England BioLabs). After terminating the digestion, the DNA was restricted with *Hin*dIII, and fragments of the appropriate size containing the hMT-II$_A$ gene 5′ flanking region were isolated on agarose gels and subcloned into the *Hin*dIII and *Hin*cII sites of pUC8 (ref. 43) to generate phMT-II-Δ3′-n, whereas n stands for the deletion end point within the hMT-II$_A$ 5′ flanking region determined by chemical sequencing[44]. Plasmids pTK-Δ5′-46 and pTK-Δ5′-109 (ref. 13) were digested with *Eco*RI (a partial digest in the case of pTK-Δ5′-109) and *Bam*HI, and the fragments containing the HSV-TK gene 5′ deletion mutants were isolated and cloned between the *Bam*HI and *Eco*RI sites of phMT-II-Δ3′-n to generate phMT-n/-46 and phMT-n/-109 respectively. There are 11 nucleotides (5′-GACGGATCCGG-3′) derived from the vector and the *Bam* linker used by McKnight that separate the HSV-TK and the MT-II gene sequences. *b*, The various phMK-n/-46 plasmids were introduced into Rat 2 cells as described in Table 1. Mixed mass cultures were derived and treated either with (+) or without (−) 5×10^{-6} Cd^{2+} before extraction of total cytoplasmic RNA. 25-μg RNA samples were analysed by Northern hybridization[45]. TK-46, parent TK gene deletion mutant used for construction of the fusions. The numbers above each set of samples denote the deletion end point within the hMT-II$_A$ regulatory region (-n1) and the deletion end point of the HSV-TK gene used (-46). Arrows, 1.3 and 0.9 kb transcripts of the HSV-TK gene.

digested with the processing exonuclease *Bal*31[11]. Different deletion derivatives were isolated by gel electrophoresis and recloned (see Fig. 1*a* for details). The progressive deletions in the 5′ to 3′ direction are referred to as phMTKΔ5′-n where n is the nucleotide present at the deletion end point within the *hMT-II$_A$* 5′ flanking region (see Fig. 1*a*). The deletion mutants were introduced into Rat 2 cells by transfection and selection in hypoxanthine-aminopterin-thymidine (HAT) medium. In the past, we have shown that selection with dexamethasone leads to 5–10-fold increase in the number of TK^+ clones generated by phMTK[10], due to increased transcriptional activity of the marker gene. This was used as a rapid assay to localize the element involved in hormonal induction. The results of such transfection experiments are shown in Table 1. Also the comparison of transfection efficiencies of the phMTKΔ 5′ mutants in the absence of hormone, helps to map regulatory elements affecting the basal level of expression.

With dexamethasone, therefore, transformation efficiency of hMTKΔ5′ mutants, containing at least 268 bp of the 5′ flanking region, is increased 5- to 14-fold. Constructs containing between 91 to 236 bp of the hMT-II$_A$ regulatory region while retaining similar basal activity are no longer hormone responsive (Table 1). Deletion mutants containing less than 91 bp of the 5′ regulatory region of the hMT-II$_A$ gene exhibit markedly reduced transformation efficiency.

Table 1 Effect of dexamethasone on transformation efficiencies of hMTK deletion mutants

Deletion end points	No. of TK^+ colonies −dexamethasone	+dexamethasone	Fold increase
Expt 1			
−585	27±2	137±4	5.1
−515	35±4	166±4	4.7
−445	26±1	118±13	4.5
−300	36±3	326±23	9.1
HSV-TK	238±7	253±11	1.06
Expt 2			
−300	28±1	220±3	8
−236	18±1	33±1	1.8
−223	31±2	28±4	0.9
−74	2±0	3±1	1.5
HSV-TK	180±6	175±7	0.97
Expt 3			
−300	22±3	114±1	5.2
−268	6±0	87±19	14.5
−91	15±3	20±3	1.3
−50	1±0.5	2±0.5	2.0
HSV-TK	110±5	140±20	1.3

Rat cells were transfected with a calcium phosphate coprecipitate of phMTKΔ5′-n or pHSV-TK (2 μg per 100 mm plate) and calf thymus carrier DNA (10 μg per 100 mm plate). Transfection and selection procedures were as previously described[10]. After 2 weeks of growth in selective medium either in the absence or in the presence of dexamethasone (10^{-6}M) the colonies were fixed, stained and counted[10]. Numbers represent averages of two or three plates.

To examine the effect of inducers on expression of the hMTK deletions, more directly, we treated stable TK^+ cultures, derived from many different individual clones with either dexamethasone or Cd^{2+} for 10 h, extracted total cytoplasmic RNA and determined the relative level of TK mRNA present. The average activity of a given mutant can be determined by examining its expression in a mixed population of cells. Individual clones vary in their levels of expression and are not suitable for such an analysis. The level of TK mRNA was increased by both dexamethasone and Cd^{2+} in all of the TK^+ cultures derived from transfection with hMTK 5′ deletion mutants containing 268 bp or more of the hMT-II$_A$ 5′ regulatory region (Fig. 1*b*). Cultures derived from deletion mutants containing 236 bp or less of 5′ flanking sequences no longer respond to the hormone, but are still metal inducible. The basal level of expression of the mutants did not vary much in most of the cultures tested, except for phMTKΔ5′–50, in which the basal level of activity of the hMT-II$_A$ promoter is essentially undetectable, however, it is still responsive to Cd^{2+}. Thus, the basal level of hMTK mRNA seems to correspond quite well to the relative transfection efficiencies of the various mutants determined in the absence of dexamethasone (see Table 1). The copy number of phMTKΔ5′-n DNA integrated into the TK^+ cells was examined and found to vary little (not more than twofold) amongst the different cultures (M.K. and A. Heguy, unpublished results.)

Next we investigated whether the deletion of 5′ flanking sequences has any effects on selection of start sites for transcription of the hMT-II$_A$ promoter. Using the S_1 nuclease mapping technique[12] we find that none of the deletions effects the choice of initiation sites for the hMT-II$_A$ promoter (Fig. 1*c*). Induction by either Cd^{2+} or dexamethasone increases the frequency of initiation at those sites.

Heavy-metal ion responsive element

Analysis of the 5′ deletion mutants indicates that only 50 nucleotides of the 5′ flanking region are necessary for heavy-metal ion induction. Our method of analysis depends on transcriptional activity for obtaining transformants and we were not successful in analysing any further deletion mutants which all fell within

the TATA box region. To achieve a better definition of the heavy-metal ion responsive element we have used a new approach to mapping of regulatory elements. To map the 3′ boundaries of such elements, *Bal*31 deletions were initiated from a unique restriction site (*Nco*I) 3′ to the promoter. In the course of mutagenesis the proximal promoter elements (TATA box and cap site) are deleted; therefore, to restore transcriptional activity hybrid promoters were constructed. The progressively deleted fragments of the hMTA-II_A regulatory region were fused to a 'crippled' HSV-TK promoter from the mutant pTK△5′-46 generated by McKnight[13]. The new hybrid promoters, therefore, contain a proximal promoter element from a gene (HSV-TK) not normally responsive to heavy metal ions[10] and various portions of the 5′ regulatory region of the hMT-II_A gene (Fig. 2*a*). The 3′ deletion mutants are referred to as phMK-n1/-46, whereas n1 stand for the deletion end point within the hMT-II_A gene. phMK-n1/-46 series plasmids (phMK-n1/-46) were introduced into Rat 2 cells. Mixed cultures were derived from many TK^+ colonies and analysed for expression of the chimaeric genes.

Surprisingly, we found that the 3′ limit of the metal responsive element lies somewhere between positions-126 to -166, since phMK-126/-46 is Cd^{2+} inducible and phMK-166/-46 is not. The pTK△5′-46 promoter itself is not responsive to Cd^{2+} and its basal level of expression is lower than any one of the hybrid promoters (Fig. 2*b*). Similar results were obtained when the 3′ deletion mutants were joined to the intact HSV-TK promoter from *pTK*△5′-109 (ref. 13), however, in this case, due to higher basal levels of expression, the fold induction after Cd^{2+} treatment was not as high (A.H., unpublished results and also see ref. 10).

In one series of experiments (Fig. 1*b*) the 5′ limit of the metal responsive element seems to lie either at or 3′ to position -50 and in another series of experiments (Fig. 2*b*) the 3′ limit mapped between -126 to -166. However, this ambiguity was settled by comparison of the nucleotide sequences between positions -30 to -50 with the sequences between -135 to -155. An extensive homology of at least 12 nucleotides was found (Fig. 3). The same nucleotide sequence is shared with three other MT genes: mouse (m) MT-I (ref. 14), rat (r) MT-I (R. Andersen, personal communication) and hMT-I_A (ref. 15).

Close examination of the level of the 1.3 kilobase (kb) transcript initiated within the proximal HSV-TK promoter (data not shown but see ref. 10) relative to the level of the 0.9-kb transcript, which is initiated from a weak promoter within the structural portion of the HSV-TK gene (ref. 16), indicated that the basal level of expression of the hybrid promoter drops between phMK-43/-66 to phMK-43/-88. Therefore, the 3′ limit of the element involved in maintaining the high basal level of expression of the hMT-II_a promoter or hybrid promoters is in between positions -66 to -88, the same region as defined earlier by the 5′ deletions.

Binding site for hormone–receptor complex

To determine whether hormonal induction of the hMTK gene is a result of binding of the hormone–receptor complex to a specific site in the gene and whether the loss of biological response to glucocorticoids is correlated with loss of receptor binding to DNA we analysed the binding of partially purified glucocorticoid receptor from rat liver[17] to the different deletion mutants. We found preferential binding of the receptor to those DNA fragments containing at least 268 bp of the region upstream of the hMT-II_A initiation site, whereas no selective binding was found to DNA fragments containing only 237 bp or less of this region (Fig. 4*a* and *b*). These findings suggest that the glucocorticoid responsive element between nucleotides -237 and -268 is also responsible for receptor binding to DNA. To define the glucocorticoid responsive sequence directly we performed DNase I protection experiments with the partially purified glucocorticoid receptor and end-labelled DNA fragments containing the hypothetical site[18,19]. This showed a

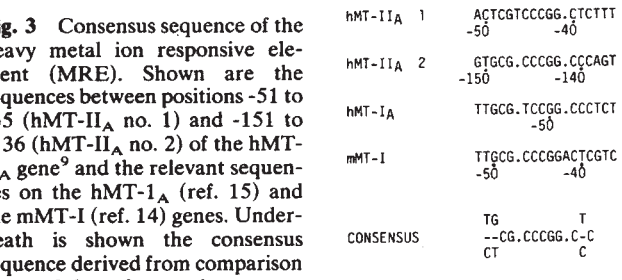

Fig. 3 Consensus sequence of the heavy metal ion responsive element (MRE). Shown are the sequences between positions -51 to -35 (hMT-II_A no. 1) and -151 to -136 (hMT-II_A no. 2) of the hMT-II_A gene[9] and the relevant sequences on the hMT-1_A (ref. 15) and the mMT-I (ref. 14) genes. Underneath is shown the consensus sequence derived from comparison of those four regions.

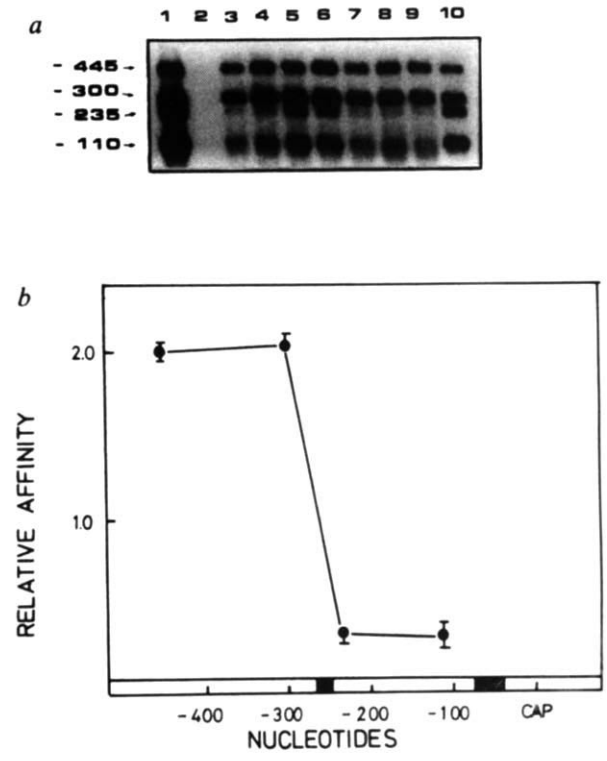

Fig. 4 Filter-binding experiments with hMT-II_A-TK deletion mutants. Four chimaeric plasmids in which the hMT-II_A-insert had been deleted up to the indicated positions upstream of the cap site (Fig. 1) were digested with *Hin*dIII and *Sst*I which cuts within the HSV-TK gene. The fragments were isolated on low-melt agarose gels, mixed in equimolar amounts and 3′ end-labelled at the *Hin*dIII site with [α-^{32}P]dATP and the large fragment of *Escherichia coli* DNA polymerase[16]. The whole length of the four fragments were 1,029 bp (deleted to -445), 884 bp (deleted to -300), 819 bp (deleted to -235) and 694 bp (deleted to -110) respectively. ***a***, An autoradiogram of the DNA fragments bound to nitrocellulose filters by the partially purified 90,000 molecular weight form of the receptor and separated by agarose gel electrophoresis. 5 ng DNA were used for each assay. Lanes 1 and 10 show the input DNA mixture. Lane 2 shows the DNA retained on the filter in the absence of receptor. Lanes 3–5 show the fragments retained at 60 mM KCl with increasing receptor concentrations (lane 3: 12 ng; lane 4: 28 ng; lane 5: 43 ng of receptor). Lanes 6 and 7 show the DNA fragments retained after incubation with 43 ng receptor at 90 mM KCl (lane 6) or 120 mM KCl (lane 7). Lanes 8 and 9 show the DNA bound to the filter by 43 ng receptor at 60 mM KCl in the presence of 25 ng native calf thymus DNA (lane 8) or 100 ng native calf thymus DNA (lane 9). ***b***, Densitometric evaluation of the autoradiogram shown in *a*. The abscissa shows the region upstream of the transcription initiation site of hMT-II_A (cap). The numbers refer to the nucleotides retained in the corresponding deletions. The ordinate represents the relative affinity, defined as the ratio between the fractional intensities of the individual bands in lanes 3, 7, 8 and 9 of the autoradiogram and the fractional intensities of the same bands in the input DNA. Average and range are shown. The black box indicates the glucocorticoid regulatory element and the cross-striped box the region required for Cd regulation.

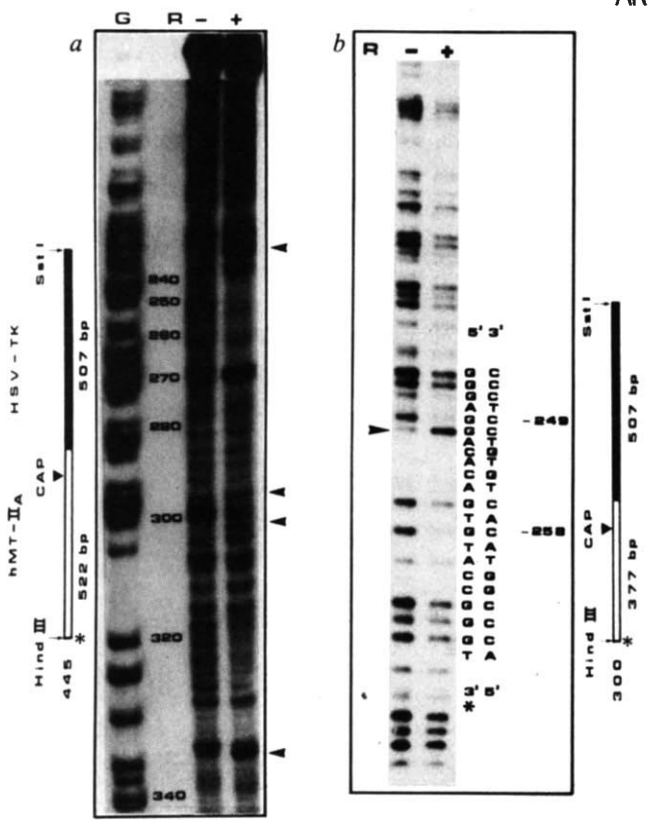

Fig. 5 An autoradiogram of a DNase I protection experiment performed with the DNA fragment shown on the left. Numbers refer to positions upstream of the cap site. The arrows indicate enhanced cutting sites of DNase I in the presence of receptor. Lane G, guanine-sequencing ladder[44]; lane R−, control DNase I ladder without receptor; lane R+, DNase I ladder in the presence of the partially purified 90,000 Mr form of the receptor. *b*, Methylation protection experiment. The figure shows the autoradiogram of a 6.5% polyacrylamide urea gel. Right, DNA fragment. Lane R−, sequence reaction specific for guanines in the absence of receptor; lane R+, the same reaction performed in the presence of the 90,000 molecular weight form of the receptor. The arrow indicates a strongly methylated guanine in the presence of receptor at position −250 from cap. The sequence of the region showing receptor-dependent changes is indicated. Numbers show the positions of two protected G residues upstream of the transcription start site. **Methods:** In *a*, the 1,029-bp *Hin*dIII–*SSt*I fragment was labelled in the *Hin*dIII site at the 3′ end with α-^{32}PdATP and the large fragment of DNA polymerase[16]. About 0.1 pmol of DNA (80,000 d.p.m.) were incubated with 56 pmol of receptor (lane R+) or without receptor (lane R−) in 180-μl assays and digested with DNase I as described[18]. Base-specific sequencing reaction for guanines (lane G) were performed with the same fragment according to Maxam and Gilbert[44]. In *b*, the DMS protection experiments were performed essentially according to Ogata and Gilbert[20]. The 884-bp *Hin*dIII–*Sst*I fragment was labelled in the *Hin*dIII site at the 3′ end with [α-^{32}P]dATP and the large fragment of DNA polymerase[16]. About 0.03 pmol of DNA (30,000 d.p.m.) were incubated with 60 pmol of receptor (lane R+) or without receptor (lane R−) in 200-μl assays containing 10 mM Tris-HCl, *p*H 7.5, 100 mM KCl, 1 mM $MgCl_2$, 1 mM Na_2EDTA, 1 mM 2-mercaptoethanol, 1 mM dithiothreitol, 1 mg ml^{-1} bovine serum albumin and 10% glycerol. After incubation for 80 min at 25 °C, followed by 5 min at 20 °C, the guanine-specific sequencing reaction[44] was performed. In control experiments we have observed that under these buffer conditions guanine methylation by DMS is indistinguishable from that detected using cacodylate buffer as recommended in the original method of Maxam and Gilbert[44].

nucleotide sequence located between -245 (±2) and -265 (±2) nucleotides upstream of the initiation site protected against DNase I digestion in the presence of the glucocorticoid receptor (Fig. 5*a*). In addition, there is a region centering at nucleotide -324 (±3) that is weakly protected against DNase I attack, whereas enhanced digestion is observed at other sites (Fig. 5*a*, arrows).

To identify further nucleotides directly involved in receptor binding we used the technique of methylation protection[20]. In this assay, contact of the receptor with specific G residues should prevent its methylation by dimethyl sulphate (DMS) resulting in disappearance of the corresponding band in a subsequent nucleotide sequencing ladder. The results obtained with the antisense strand are shown in Fig. 5*b*. At least two G residues, at positions -249 and -258, participate in the interaction with the receptor. A third G residue exhibits enhanced methylation in the presence of the receptor (Fig. 5*b*, arrow). No other changes in the guanine methylation pattern were induced by the receptor in the region between -300 and the initiation site of the hMT-II_A gene.

Discussion

Our results indicate that binding of the purified glucocorticoid hormone–receptor complex to a defined site within the 5′ flanking region of the hMT-II_A gene leads to increased transcription from a promoter located 250 bp away. A separate regulatory element present twice within that control region is involved in the induction response to heavy metal ions. Both of these elements can activate heterologous promoters located at least 600 bp away, suggesting that enhancement of transcription by both glucocorticoid hormones and heavy metal ions is mediated via the activation of 'enhancer-like' elements[10].

The analysis of a series of *in vitro* constructed 5′ deletion mutants indicates that at least 268 bp derived from the 5′ flanking region of the hMT-II_A gene are required for glucocorticoid hormone mediated induction. Deletion mutants retaining 237 to 50 bp of the regulatory region are no longer hormone inducible and respond only to heavy metal ions. Therefore, we conclude that an upstream control element situated between nucleotides -268 to -237 of the hMT-II_A gene is required for hormonal induction and a different control element is important for heavy metal induction. The metal ion responsive element is a dodecameric sequence, highly conserved between all functional MT genes whose sequence is known (Fig. 3), and is present twice within the 5′ flanking region of the hMT-II_A gene. While we cannot rule out synergistic or cooperative relationships between the two copies of the element, located between -38 to -50 and -138 to -150 on the hMT-II_A gene, it is clear that a single copy can confer heavy metal ion responsiveness upon various proximal promoter elements.

We have identified the binding site of the glucocorticoid hormone receptor complex on the hMT-II_A 5′ flanking region by testing the receptor binding capacity of the various 5′ deletion mutants and by protection against DNase I digestion and DMS methylation, due to receptor binding ('foot-printing'). This binding site is located between nucleotides -265 (±2) to -245 (±2) of the human gene.

Taken together, these results demonstrate unequivocally that the same DNA region (-265 to -245) acts both as a regulatory element necessary for glucocorticoid induction of transcription, defined by a reversed genetic approach, and as a binding site for the hormone–receptor complex, defined by the DNA binding studies. Therefore the *in vitro* determined binding site is probably identical to the physiological site of interaction between the receptor and the gene. Moreover, since the deletion of that site does not effect the basal level of phMT-II_A promoter activity or its ability to be induced by heavy metal ions, it appears that the glucocorticoid hormone–receptor is a positive regulatory factor. This is the first time such a relationship has been clearly established. Similar results, have recently been obtained by several groups investigating both hormone–receptor binding and expression of the long terminal repeat (LTR) of mouse mammary tumour virus (MMTV)[17,19,21–23]. However, due to certain inherent limitations of the MMTV system, the relationship between receptor binding sites determined *in vitro*, and effects of hormone on transcription determined *in vivo* is not as clear as for the hMT-II_A promoter. Due to very low level of basal promoter activity in the absence of hormone, it is hard to dissociate the effects of deletions on inducibility of the viral

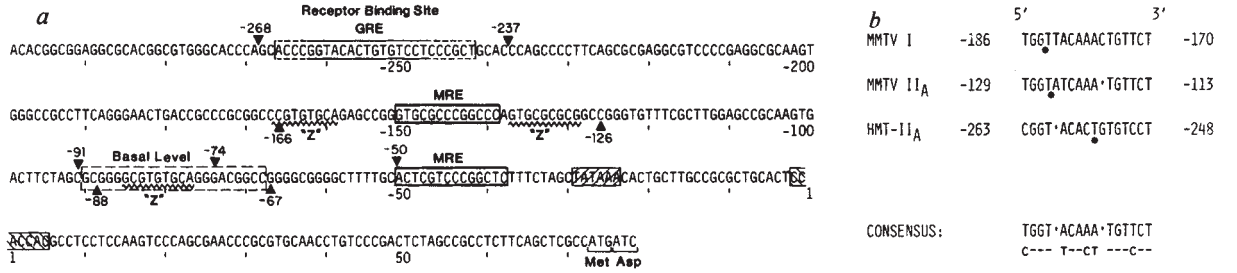

Fig. 6 *a*, Molecular anatomy of the *hMT-II*$_A$ promoter. Sequence of the sense strand of DNA between positions −300 and +80. Regions important for hormonal induction (GRE for glucocorticoid responsive element), heavy metal induction (MRE for metal ion responsive element), and basal level of expression. Glucocorticoid regulatory element: solid line, the region protected by binding of the receptor to DNA; broken line, uncertainties in exact borders of the region. Also indicated are the TATA box and the cap site (hatched boxes) and region of alternating purine–pyrimidine sequences (Z-DNA), and the first two amino acid codons of the translated region. Arrowheads and numbers indicate the positions of deletion end points important for the mapping of the various regulatory elements. Arrowheads above the line indicate 5′ deletions, and below the line 3′ deletions. *b*, Consensus sequence for the glucocorticoid receptor binding to DNA. The sequence designated *MMTV*I and *MMTV*II$_A$ correspond to regions in the LTR of MMTV strongly protected by the receptor against DNase I digestion[19]. The *hMT-II*$_A$ sequence is taken from Fig. 4*a* and ref. 9. The asterisks mark the symmetry centre of small palindromes.

promoter, from an effect on its basal transcriptional activity[21,23]. Also, the small, but significant induction observed in an MMTV deletion mutant containing only 50 bp of 5′ flanking DNA[21] and presumably no receptor binding sites[18,19] leads to further confusion.

Comparison of the hMT-II$_A$ gene nucleotide sequence at the region between -267 and -243 (ref. 9) to the MMTV-LTR sequences between -191 to -170 and -132 to -113 that are strongly protected against DNase I digestion[19] reveals a high degree of nucleotide sequence homology. We propose that the conserved sequence elements between those three different binding sites (shown in Fig. 6*b*) make up the consensus nucleotide sequence for glucocorticoid hormone–receptor binding to DNA and consequently hormonal induction. Final confirmation, however, will depend on the outcome of experiments testing the biological activity of synthetic consensus sequences. Interestingly, a sequence which is in complete agreement with our proposed consensus sequence is found within the first intron (at position +95 to +109) of the human growth hormone gene[24], a gene which is induced by dexamethasone after transfection[25]. Preliminary results indicate that this sequence functions as a receptor binding site (D. Moore, personal communication).

A weaker binding of the receptor to DNA is observed when the hexanucleotide 5′-TGT$\frac{T}{C}$CT-3′ occurs isolated from the rest of the consensus sequence, as for instance between -70 and -100 in MMTV–LTR or at -324 in the antisense strand of hMT-II$_A$ (Figs 4*a* and 5*a*). Since, however, this region of the hMT-II$_A$ gene can be deleted without influencing the extent of hormonal induction, it seems that at least in this case binding of the receptor to the hexanucleotide is not functionally relevant.

Also we note that binding of receptor to the hexanucleotide results in a protected region covering only 12 bp, while binding to the complete consensus sequence protects 24 bp (Fig. 5*a* and ref. 19). It is likely that a dimer of the receptor is involved in generating a biological response. Methylation protection experiments suggest that at least two G residues located at -249 and -258 participate in the interaction of the receptor with its binding site. Since these residues are separated by 9 bp they should be located on the same side of the DNA helix. In addition, DMS methylation is selective for the N-7 position of G residues located in the DNA major groove[26]. Thus, our results suggest that the receptor contacts at least one face of the DNA helix via the major groove.

We believe that a third control element is located between nucelotides -90 and -70, since 5′ and 3′ deletion mutants lacking this region exhibit reduced basal level of expression. A requirement for certain upstream nucleotide sequences, not directly involved in heavy metal induction, but which are important for maintenance of basal level of expression was seen by Palmiter and Hamer and co-workers (ref. 27. P. Searle; A. Carter and D. Hamer, personal communications) studying the mMT-I gene by various transient expression assays.

Interestingly, the region between nucleotides -70 to -90 contains a stretch of alternating purine–pyrimidine residues: GCGTGTGCA and a similar sequence is located 80-bp upstream: CGTGTGCA. Such sequences capable of forming a Z–DNA structure have been found in a large number of transcriptional enhancer sequences from a variety of DNA and RNA tumour viruses[28]. A functional role for these elements is strongly suggested by the analysis of the transcriptional activity of the deletion mutants. While deletion of the upstream segment does not have a significant effect on the basal level of gene expression, deletion of both elements dramatically reduces the basal activity of the hMT-II$_A$ promoter.

The overall organization of the hMT-II$_A$ gene regulatory region resembles that of several other eukaryotic genes[29–34]. In addition to the TATA box region, a functional element common to most eukaryotic genes[35], those genes were found to have at least two other functional elements. One of them is usually located near the TATA box region, and the other located further upstream is in the -90 region. In addition, further upstream elements required for efficient transcription have been described for the sea urchin H2A gene[36] and the simian virus 40 early region[32,34,37].

As several inducible higher eukaryotic genes have now been analysed by *in vitro* mutagenesis and gene transfer, some conclusions concerning control of transcription can be drawn. First, unlike prokaryotic promoters, the eukaryotic promoter is extended over a large region composed of different functional elements (270 bp in the case of the hMT-II$_A$ gene). Second, the comparison of functionally related control elements reveals the conservation of nucleotide sequences among such elements (refs 38–40 and this study). The conservation of nucleotide sequences among functionally related regulatory elements suggests that those elements serve as binding sites for various regulatory proteins. The identity of most regulatory proteins involved in the various induction phenomena mentioned above, is not known yet. However, our current study and recent work on MMTV–LTR[19,21–23] have established that one such protein, the glucocorticoid receptor, interacts directly with regulatory sites important for the biological response.

How is this interaction translated into increased transcription (or repression in some cases)? The exact molecular details are not clear yet, but recent work on the regulatory elements of hMT-II$_A$ gene[10], the MMTV–LTR (refs 21–23) and of the *Hsp*70 heat shock gene[39,40] indicates that those elements can activate heterologous promoters when placed at various distances upstream. This feature bears some resemblance to the

effects of a better known class of regulatory elements, the viral 'enhancers'[32,34,37,41]. It is quite possible that the various 'modulator' elements, described, can serve as regulated RNA polymerase entry sites. Another possibility, which we favour, is that the interaction of regulatory proteins with the DNA at upstream sites leads to a change in the conformation of the double helix, a signal that can be transmitted over some distance, and lead to local melting at the TATA box region, the site for RNA polymerase and initiation factors binding to DNA[42].

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LETTERS TO NATURE

X-ray emission from non-magnetic cataclysmic variables

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Many (~50) cataclysmic variables, binary systems in which a white dwarf accretes from a close companion star, are now known to be hard (≥2 keV) and variable X-ray sources[1]. While some of these (~15) exhibit regular pulsing in X rays, suggesting that they have magnetic fields strong enough to channel the accretion flow onto restricted parts of the white dwarf surface (the AM Her and 'intermediate polar' systems), the majority do not, and hence presumably have rather weaker fields ($B \leqslant 10^5$ G). SS Cygni, the brightest object of this 'non-magnetic' class, exhibits a combination of systematic features in its X-ray and optical emission which existing models are only partially successful in reproducing. We suggest here that at sufficiently low accretion rates a thermal instability in the accretion flow leads to the formation of a hard X-ray-emitting corona around the white dwarf; the corona is relatively suppressed at higher accretion rates. We show that the cooling of such a corona gives the observed correlation between X-ray temperature and luminosity, and that hard X-ray production may be rather inefficient because of transport processes. These can give rise to soft X-ray emission from most of the white dwarf surface, with a correspondingly low effective temperature.

Any theoretical model for the hard X-ray emission from these systems must explain three key observational facts: (1) SS Cygni is a bright and very variable hard X-ray source in quiescence (that is, low accretion rates $\dot{M}$), but the hard X rays are severely reduced during optical outbursts[2] (increased accretion rates) while the soft X rays brighten[1,2]. (2) The drop in hard X-ray intensity of SS Cygni is not accompanied by an increase in intrinsic (for example, photoelectric) absorption[3]. (3) Hard X-ray spectra of SS Cygni are thermal, with bremsstrahlung temperature $kT \leqslant 20$ keV[1]; these are strongly correlated (J. H. Swank, cited in ref. 1 and M. J. Ricketts and A. Bennetts, personal communication) with X-ray luminosity L_x, T and L_x rising and falling together.

The only plausible energy reservoir for hard X-ray production is the boundary layer where the inner edge of the accretion disk interacts with the white dwarf. If this has mass and radius M, R_*, the keplerian kinetic energy which must be lost by the boundary-layer material before accreting to the white dwarf (assumed to be rotating below break-up velocity) provides a luminosity $GM\dot{M}/2R_*$. Existing models[4,5] of hard X-ray production invoke optically thin emission from the boundary layer; if the boundary layer is optically thick, soft X rays at an effective temperature $kT \sim 5$–10 eV result[6]. Refs 4, 5 both explain fact (1) above in terms of increased absorption, and hence fall foul of fact (2). While Tylenda's model[5] gives no natural explanation for fact (3), we shall show that this is likely to follow for any model in which the X-ray-emitting gas can expand to form a corona around the white dwarf before cooling. This is the case in Pringle and Savonije's model[4] for low $\dot{M}$.

We consider an optically thin element of boundary-layer gas of radial extent $b < R_*$, orbiting the white dwarf with azimuthal velocity $v_\phi \sim v_k = (GM/R_*)^{1/2}$ (by definition $v_\phi < v_k$ in the boundary layer). Because of its high rate ($\sim v_\phi/b$) of shear, we expect the boundary-layer gas to be very turbulent; this turbulence will both decelerate and heat the orbiting gas. We write

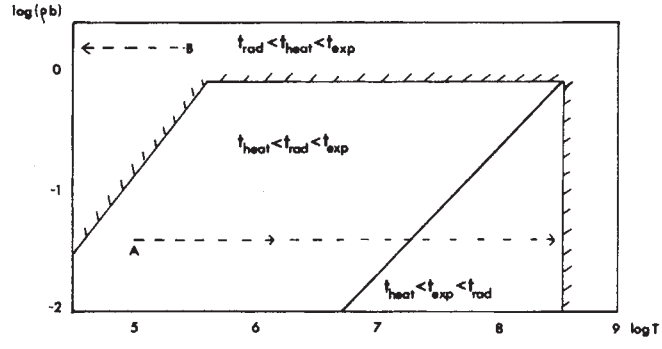

Fig. 1 Heating plane for the case $M = M_\odot$, $R = 5 \times 10^8$ cm, $\alpha = 1$, $(v_\phi/v_k)^2 = 0.5$. Gas elements characterized by initial points lying in the hatched region heat up to X-ray temperatures.

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