shell pattern by periostracum is obvious. The development of the funnel, tentacles, and hood will require further analysis before conclusions can be drawn regarding the relation of these organs to the head and foot.


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# In Vivo Competition Between a Metallothionein Regulatory Element and the SV40 Enhancer


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**The human metallothionein-II$_A$ (hMT-II$_A$) gene contains an enhancer element within its 5′ regulatory region. This enhancer element can compete with the SV40 enhancer for one or more cellular factors in vivo. The competition between the two elements is modulated by cadmium, an inducer of hMT-II$_A$ transcription. The data presented are consistent with a model in which heavy metal ions control the ability of the hMT-II$_A$ enhancer to bind a positive factor, leading to increased transcription. The same factor is required for maximal activity of the SV40 enhancer, which suggests that viruses utilize factors that have a normal role in cellular gene expression to control their own genes.**


EXPRESSION OF EUKARYOTIC GENES transcribed by RNA polymerase II is controlled by a variety of *cis*-acting genetic elements, including promoters (*1*), enhancers (*2, 3*), and responsive elements (*4–6*). These *cis*-acting elements are thought to serve as binding sites for *trans*-acting regulatory proteins. However, with few exceptions, including T antigen (*7*), heat shock transcription factor (*8*), SP1 (*9*), and the glucocorticoid (*6, 10*) and progesterone (*11*) hormone receptors, none of these factors or their interactions with DNA have been characterized.

The human metallothionein-II$_A$ (hMT-II$_A$) gene (*12*) can be induced by heavy metal ions and glucocorticoid hormones. This induction (and control of the basal level of the gene's expression) are regulated by elements within the 5′ flanking region of the hMT-II$_A$ gene (*6*). Similar elements have also been described for the mouse (m)MT-I gene (*13*). These regulatory elements can activate heterologous promoters in a distance-independent manner (*6, 14*), a property shared with enhancer elements (*2*). We have characterized an enhancer element, present within the hMT-II$_A$ 5′ regulatory region (*15*), whose activity is further increased in the presence of heavy metal ions such as cadmium ($Cd^{2+}$) (*16*).

Recently, Scholer and Gruss (*17*) developed a novel approach that allows further insight into the mechanisms that control gene expression, in the absence of detailed knowledge of the regulatory factors involved. By means of an in vivo assay, they have demonstrated competition between enhancer-containing molecules derived from SV40 and murine sarcoma virus for cellular targets. Tissue-specific competition has been demonstrated between immunoglobulin and SV40 enhancers (*18*). All of these enhancers contain a common sequence known as the "core" that is important for their function (*19*). We now show that regulatory elements of the hMT-II$_A$ gene are capable of competing with the enhancer element of SV40 (and vice versa) for a common cellular target and that the competition is modulated by $Cd^{2+}$.

To understand how $Cd^{2+}$ regulates the activity of the enhancer element we have used in vivo competition experiments, since thus far this phenomenon cannot be studied in an in vitro system. Because these experiments require transient expression of deletion mutants of hMT-II$_A$ in primate cells and our original characterization of the hMT-II$_A$ regulatory region was based on stably transformed rat fibroblast lines, we wished to redefine some of the nucleotide sequence requirements for hMT-II$_A$ expression under conditions more similar to those of the competition assay. Deletion mutants of the hMT-II$_A$ 5′ flanking region (*6*) were fused to the bacterial gene coding for chloramphenicol acetyltransferase (CAT; *20*) (Fig. 1A) and were transfected into HeLa and CV-1 cells. Deletion of hMT-II$_A$ 5′ flanking DNA from the 5′-most Hind III site to position −160 relative to the start of transcription had no effect on either the basal or induced level of CAT expression (Fig. 1B). The fusion genes containing at least this amount of 5′-flanking DNA were induced 7- to 10-fold by $Cd^{2+}$. The extent of induction in different experiments ranged between 3- to 12-fold due to fluctuations in the basal level; however, within a single experiment, the response was much less variable. A deletion to position −96 decreased the basal level of promoter activity by almost one order of magnitude, but the mutant was still responsive to $Cd^{2+}$. A further deletion to position −50 abolished expression altogether. No significant differences were observed between HeLa and CV-1 cells with respect to expression of the fusion genes except for the higher transfection efficiency of the latter cell line.

In stably transformed Rat 2 cells, a deletion to position −96 had no significant effect on the basal level of promoter activity


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and a deletion to position −50, although leading to complete abolition of basal expression from the promoter, was still responsive to $Cd^{2+}$ (*6*). However, we have observed that analysis of these mutants by a transient assay in Rat 1 cells yields similar results to those obtained in HeLa cells, indicating that there are some intrinsic differences between analyzing hMT-$II_A$ gene expression by a transient assay and by the use of stable transformants directly selected for expression of a MT-TK fusion gene. Since no selective pressure is applied in the transient assay, its results are more likely to reflect the natural requirements for transcription factors. A similar requirement for sequence elements whose 5′ border is at −151 was found for the mMT-I gene transfected into CV-1 cells (*13*). The amount of DNA did not affect the ability of $Cd^{2+}$ to induce CAT activity in the MT-CAT fusions (Fig. 1C). Expression of CAT under the control of the SV40 early promoter and enhancer (pSV2CAT), on the other hand, is not significantly affected by $Cd^{2+}$.

Thus, while the activity of the SV40 element was not modulated by $Cd^{2+}$, the activity of the hMT-$II_A$ promoter/regulatory elements was increased up to 12-fold in the presence of $Cd^{2+}$. To determine whether the two elements interact with a common cellular target, and whether this interaction is affected by $Cd^{2+}$, we used an in vivo competition assay. A constant amount of pMTCATΔ5′-286 was transfected into cultured cells in the presence of increasing amounts of the competing DNA, pSV2Neo, in which the SV40 enhancer and early promoter control the expression of a bacterial gene coding for aminoglycoside resistance (*21*). In the absence of $Cd^{2+}$, pSV2Neo competed very efficiently with pMTCATΔ5′-286 (Fig. 2). A 2.4-fold molar excess of the plasmid containing the viral enhancer was sufficient to reduce CAT expression to 10 percent of its basal level. In the presence of $Cd^{2+}$, pSV2Neo competed less efficiently and a 30-fold molar excess was required to achieve a similar level of competition. No competition was observed when pSV0Neo, which does not contain the SV40 enhancer, was used as the competitor. Since the activity of the viral promoter and enhancer is not affected by $Cd^{2+}$ (Fig. 1), these results suggested that the heavy metal ion modulates the ability of the hMT-$II_A$ promoter/regulatory region to bind a factor that is also recognized by the SV40 enhancer.

The very efficient inhibition of hMT-$II_A$ promoter activity by pSV2Neo suggested that the SV40 enhancer has a higher relative affinity for the limiting factor than the hMT-$II_A$ promoter in the absence of heavy metal

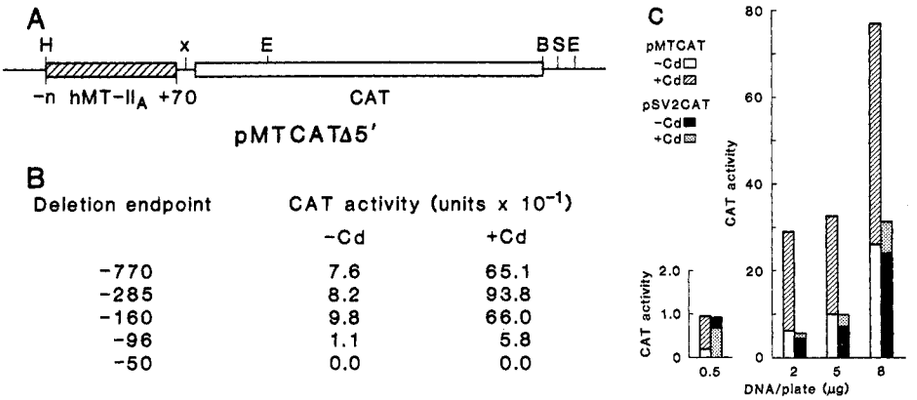


Fig. 1. Expression of MT · CAT deletion mutants. (A) Structure of MT · CATΔ5′ fusion genes. A Bgl II–Bam HI fragment from pSV0CAT (*20*), which contains the structural sequences, intron, and 3′-flanking region of the CAT transcription unit, was subcloned into the Bam HI site of pUC8 to generate the vector pUCAT(−). The hMTKΔ5′ plasmids (*6*) were linearized with Nco I (position +73 in the hMT-$II_A$ 5′-leader), digested with S1 nuclease and then with Hind III; the fragments containing hMT-$II_A$ 5′-flanking DNA were subcloned between the Hind III and Hinc II sites of pUCAT(−) to generate the pMTCATΔ5′ series. The 5′ deletion endpoint is indicated by −n. (B) Expression of pMTCATΔ5′. A calcium phosphate coprecipitate containing pMTCATΔ5′ (10 μg) was added to a monolayer culture of HeLa cells ($10^6$ cells) for 4 hours. After an incubation for 2 to 3 minutes in the presence of 15 percent glycerol, fresh medium was added with or without $5 \times 10^{-6} M$ $CdCl_2$. After 12 to 14 hours, the cells were harvested, and lysates were assayed for CAT expression (*15, 20*). Average values of two separate determinations of CAT activity (pmoles of chloramphenicol acetylated per μg of protein per hour are shown. The variation between duplicates was no greater than ±10 percent. (C) CAT expression from pMTCATΔ5′-770 (pMTCAT) and pSV2CAT as a function of DNA concentration. HeLa cells were transfected with the indicated amounts of each plasmid plus pUC8 DNA, which was used to bring the total amount of DNA to 10 μg. The values represent averages of two separate determinations, varying by less than ±10 percent.

ions. Therefore, we performed a competition assay between pMTCATΔ5′-286 and pMTKΔ5′-286 (Fig. 2). The hMT-$II_A$ control region did have a relatively lower affinity toward the limiting factor than the SV40 enhancer. This conclusion is also supported by our finding that at low concentrations of transfected DNA (0.5 μg per plate) the basal activity of pMTCAT was lower than that of pSV2CAT, while at higher concentrations the two activities were essentially equal (Fig. 1C).

To localize the sequence(s) within the hMT-$II_A$ regulatory region required for competition, we have performed additional competition experiments in which the indicator gene was pSV2CAT (*20*) and competitor DNA's were various MT–thymidine kinase (TK) fusion genes. Under these conditions, no competition was observed in the absence of $Cd^{2+}$, even at very high levels of competitor DNA. However, in the presence of $Cd^{2+}$, a 40-fold molar excess of plasmids that contain hMT-$II_A$ sequence elements between position −67 and −226 resulted in a decline in CAT activity to 35 to 40 percent of its initial level (Fig. 3A). Deletions that extended into this region led to a decrease in the observed competition.

To verify that the measured CAT activity reflects initiation from the SV40 early promoter and that treatment with $Cd^{2+}$ has no effect on the accuracy of initiation, the start sites of CAT and TK messenger RNA (mRNA) were determined by primer extension. In the absence or presence of $Cd^{2+}$, the SV · CAT and the MTK transcripts all initiated from their correct start sites (*12, 22, 23*), and only the latter were induced by the metal ion (Fig. 3B).

The region of the hMT-$II_A$ gene between positions −67 to −226 that is important for competition (Fig. 3A) contains two imperfect direct repeats important for its enhancer activity (*15*). To further localize the elements responsible for the observed competition we have tested various plasmids containing different configurations of the proximal (−67 to −140) and distal (−140 to −214) repeats as competitors. Both repeats were similar in their competition activity (Fig. 4), indicating that each repeat is likely to contain a binding site for the competed factor. No competition was observed between the HSV-TK gene and pSV2CAT, even though the activity of both of their promoters is dependent on binding of a common factor, SP1 (*9, 24*). This result also indicates that the observed competition is not for factors that bind to the TATA box region (*25*) and RNA polymerase II, which are required by all promoters.

The results of the competition experiments suggest that the hMT-$II_A$ and SV40 enhancer elements compete for a common cellular factor(s) required for their activa-

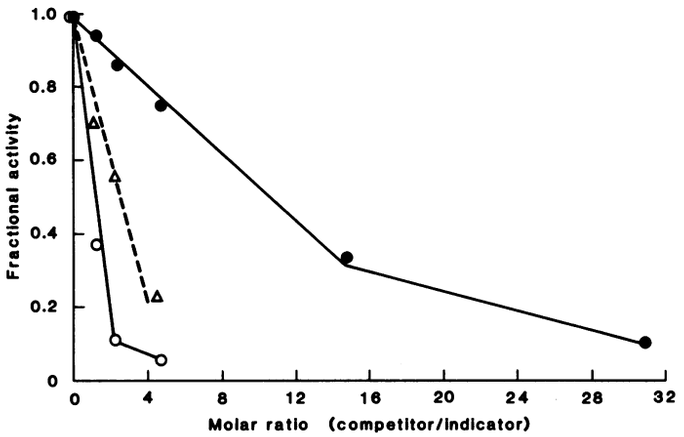


Fig. 2. In vivo competition between SV40 and hMT-II$_A$ regulatory elements. A constant amount of the indicator plasmid pMTCATΔ5′-286, previously determined to be optimal for expression, was co-transfected with increasing amounts of the competitor plasmid, pSV2Neo, into CV-1 cells. Carrier DNA (pUC8) was used to keep the total amount of DNA constant. The cells were incubated with the DNA-$CaPO_4$ coprecipitate for 4 hours, glycerol-shocked, and then incubated for another 12 to 14 hours in normal growth medium with or without $5 \times 10^6 M$ $Cd^{2+}$. CAT activity was determined in duplicate and average values of three different experiments varying by less than ±10 percent are expressed as fractional activity (activity in the presence of competitor÷activity in the absence of competitor). The amount of indicator was 4 μg for molar ratios up to 16 and 0.75 μg for higher molar ratios. Competition with pSV2Neo in the absence of $Cd^{2+}$ (○); competition with pSV2Neo in the presence of $Cd^{2+}$ (●); competition with pMTKΔ5′-286 in the absence of $Cd^{2+}$ (△).

tion. While the apparent affinity of the viral enhancer for this factor(s) is high, the hMT-II$_A$ enhancer does not bind as efficiently. Furthermore, the interaction of the hMT-II$_A$ regulatory elements with this factor(s) is regulated by $Cd^{2+}$.

The region of hMT-II$_A$ DNA that is important for the competition and possibly for binding of the factor(s) lies between positions −67 to −226 and is composed of two direct repeats, both of which are important for activity of the hMT-II$_A$ enhancer element (*15*). The repeats contain several common core sequences important for basal level of expression commonly referred to as BLE's (basal level elements) and one or two copies of a metal-responsive element (MRE) (*6, 16*). The proximal repeat contains two regions of homology to the distal repeat (−96 to −130 and −78 to −96) that are important for expression of the hMT-II$_A$ promoter (Fig. 1B). Deletions that extend into the BLE's (Δ5′-96, Δ5′-80, and Δ3′-126) also reduced the competitor activity of the hMT-II$_A$ regulatory region. However, two of these mutants, Δ5′-96 and Δ3′-126, were still inducible by $Cd^{2+}$ (Figs. 1B and 3) (*6*). These results suggest that the competition is for a factor that recognizes the BLE's, and not the MRE.

This interpretation is supported by the lack of response of the SV40 early promoter to metal induction and the absence of a sequence homologous to an MRE within it. However, several sequences similar to the BLE's can be found in the viral element

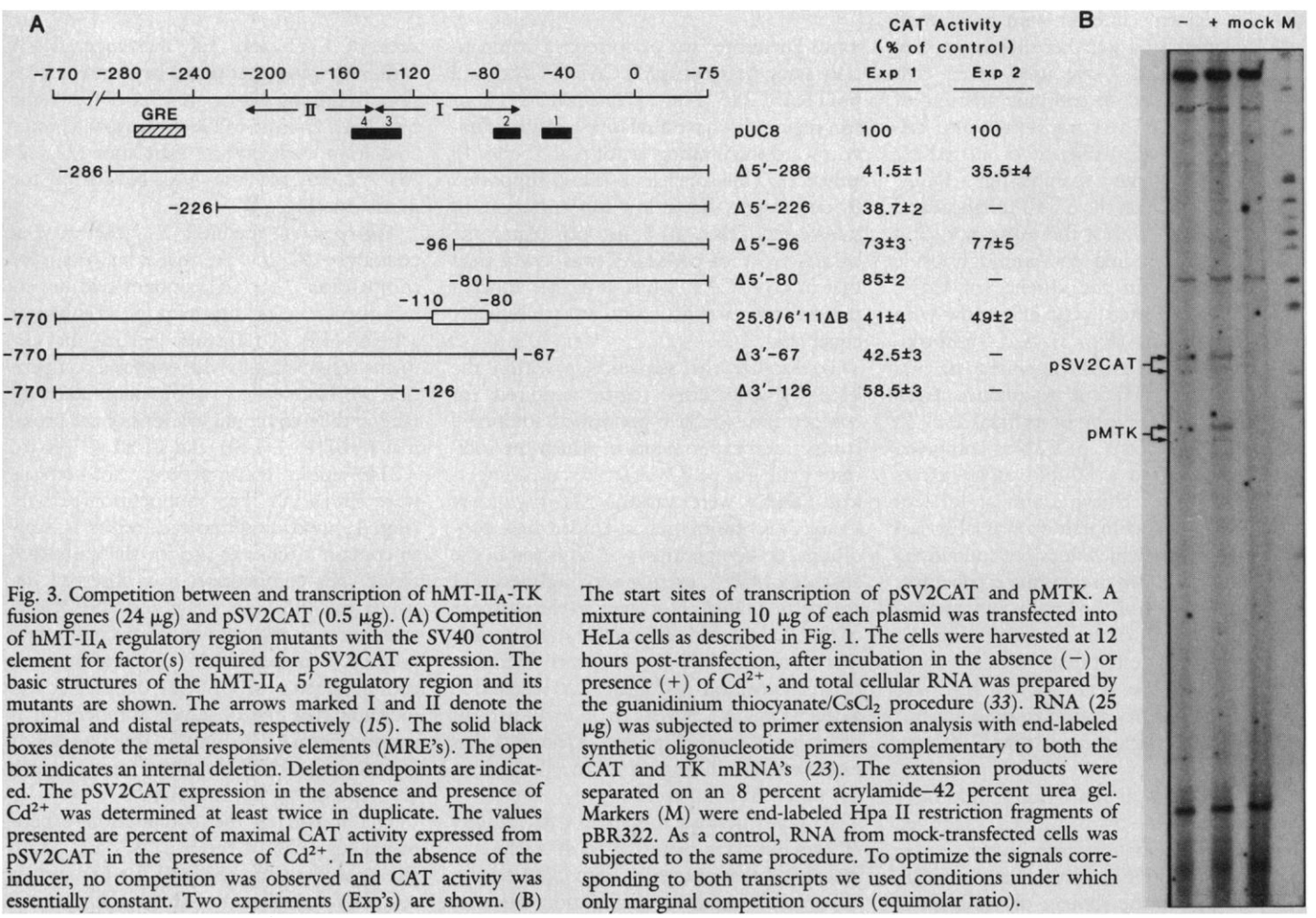


Fig. 3. Competition between and transcription of hMT-II$_A$-TK fusion genes (24 μg) and pSV2CAT (0.5 μg). (A) Competition of hMT-II$_A$ regulatory region mutants with the SV40 control element for factor(s) required for pSV2CAT expression. The basic structures of the hMT-II$_A$ 5′ regulatory region and its mutants are shown. The arrows marked I and II denote the proximal and distal repeats, respectively (*15*). The solid black boxes denote the metal responsive elements (MRE's). The open box indicates an internal deletion. Deletion endpoints are indicated. The pSV2CAT expression in the absence and presence of $Cd^{2+}$ was determined at least twice in duplicate. The values presented are percent of maximal CAT activity expressed from pSV2CAT in the presence of $Cd^{2+}$. In the absence of the inducer, no competition was observed and CAT activity was essentially constant. Two experiments (Exp's) are shown. (B) The start sites of transcription of pSV2CAT and pMTK. A mixture containing 10 μg of each plasmid was transfected into HeLa cells as described in Fig. 1. The cells were harvested at 12 hours post-transfection, after incubation in the absence (−) or presence (+) of $Cd^{2+}$, and total cellular RNA was prepared by the guanidinium thiocyanate/$CsCl_2$ procedure (*33*). RNA (25 μg) was subjected to primer extension analysis with end-labeled synthetic oligonucleotide primers complementary to both the CAT and TK mRNA's (*23*). The extension products were separated on an 8 percent acrylamide–42 percent urea gel. Markers (M) were end-labeled Hpa II restriction fragments of pBR322. As a control, RNA from mock-transfected cells was subjected to the same procedure. To optimize the signals corresponding to both transcripts we used conditions under which only marginal competition occurs (equimolar ratio).

(*15*). Neither the MRE nor the BLE [in constructs containing isolated, synthetic elements (*26*)] competed with pSV2CAT for factor binding by themselves (Fig. 4). However, while the MRE is the only sequence important for induction by $Cd^{2+}$ (*6, 13*), the synthetic BLE is not the only sequence important for basal activity (*16*) (Fig. 1). Therefore, the inability to observe competition with the isolated BLE may indicate either that this element does not contain the binding site for the limiting factor or that it is not active by itself because it requires cooperative interactions with other regulatory elements involved in basal expression or with the MRE's.

Competition has been detected between two mMT-I fusion genes for a common cellular factor required for their maximal activity (*27*). The sequences important for the competition were between −151 and −102, yet, in their absence, the mMT-I gene was still inducible by $Cd^{2+}$ (*13*). No competition was observed between mMT-I and SV40. This possible disagreement with our results could be due to the use by Seguin *et al.* (*27*) of replicating SV40 vectors. Under such conditions it could be difficult to observe competition for factors required for expression of the SV40 early promoter since promoter activity also controls the copy number of the plasmids by expression of T antigen.

If the MRE itself does not serve as a binding site for the competed factor, how do metal ions control the competition activity? A likely explanation is that a metal regulatory factor (MRF) that binds to the MRE modulates the interaction of a rate-limiting factor with the BLE. At least two models can be proposed to account for such an interaction. According to the first model, the MRF stabilizes the binding of the limiting factor to the BLE in the presence of heavy metal ions via protein-protein interaction. This would explain the failure to observe competition with an isolated BLE. In the second model, the MRF acts as a repressor in the absence of $Cd^{2+}$ that binds to the MRE and interferes with the binding of the limiting factor to the adjacent positive regulatory site (BLE). In the presence of $Cd^{2+}$, the MRF no longer binds to DNA and repression is relieved. While both of these models take into account competition for a single factor, we cannot exclude the possibility that the SV40 and the hMT-$II_A$ enhancers compete for more than one factor. In fact, each of these elements contains sites for at least three different regulatory proteins (*28*).

The hMT-$II_A$ enhancer, like that of the immunoglobulin genes, is a conditional enhancer. While the immunoglobulin enhancer is active only in lymphoid cells (*3*), the hMT-$II_A$ enhancer requires $Cd^{2+}$ for its full activity (*16*). Other conditional enhancers include glucocorticoid-responsive elements (GRE's), which are activated by binding of the glucocorticoid hormone receptor (*4, 6*), and the enhancers of several leukemia viruses, which seem to require a viral-encoded function for maximal activity (*29*). Like the hMT-$II_A$ enhancer, the immunoglobulin enhancer competes with the SV40 enhancer only after it is fully activated by binding of factor(s) present in lymphoid cells (*18*). These similarities suggest that the activity of enhancer elements is regulated by *trans*-acting factors that interact with each other. Some of these factors are not common to several enhancers, but seem to be responsible for conferring cell-type and induction specificity.

It is too early to tell whether the competition observed between SV40 and hMT-$II_A$, or SV40 and immunoglobulin enhancers, is for the same factor. The hMT-$II_A$ regulatory region contains various sequences found in the SV40 enhancer, including a sequence similar to the core and to stretches of alternating purine-pyrimidine residues [a common feature in many viral enhancer elements (*30*)]. These sequences are important for the activity of the SV40 enhancer (*28*). Another common feature of the hMT-$II_A$ and the SV40 enhancers is a sequence that is homologous to a consensus of adenovirus en-

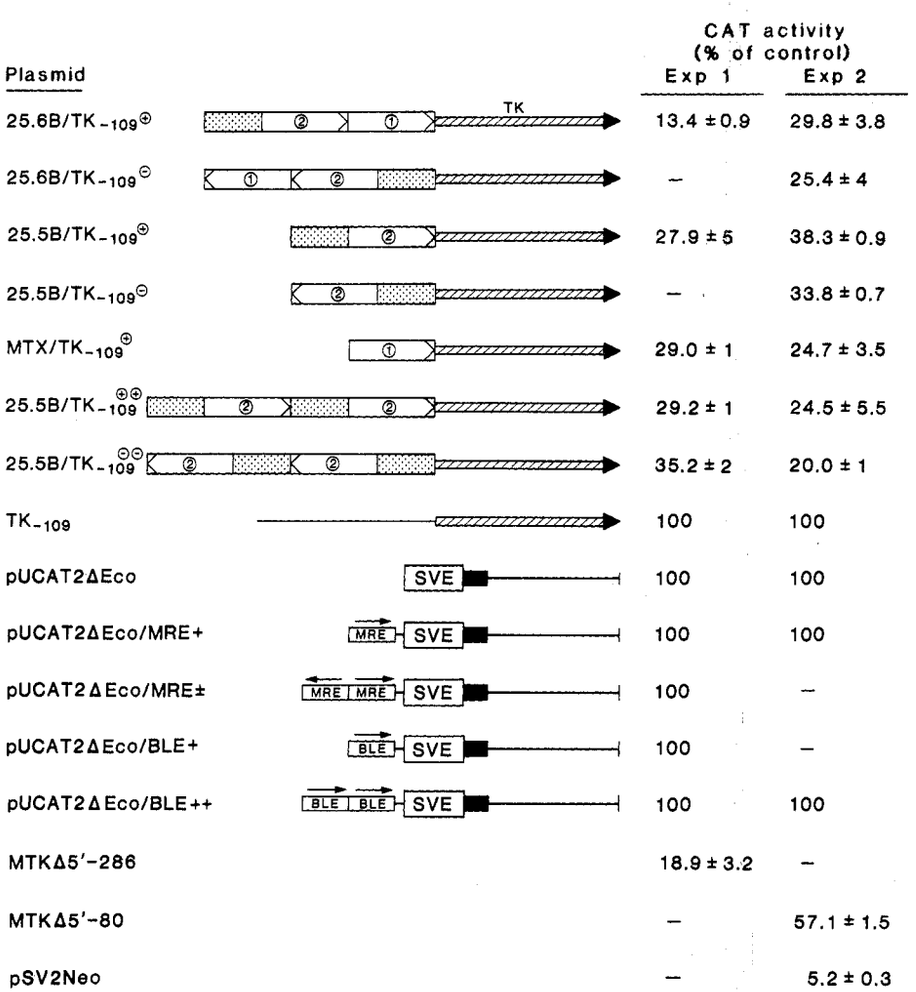


Fig. 4. The hMT-$II_A$ regulatory region repeats are effective competitors. MT/TK plasmid constructs (*15*) contained DNA fragments with both of the hMT-$II_A$ regulatory region repeats (−69 to −335), the proximal repeat (open-box 1, −67 to −132), or the distal repeat (open-box 2, −130 to −335), inserted into the Bam HI site of pTKΔ5′-109 (*34*) with Bam HI linkers. The orientation of the hMT-$II_A$ repeats is indicated by the arrowheads. The dotted boxes represent hMT-$II_A$ sequences between −214 to −335, which are not part of the repeats and are not required for competition (Fig. 3) or expression (Fig. 1). The + and − refer to the orientation of the MT-regulatory sequences; the number of symbols refers to the number of inserts. Synthetic MRE and BLE elements (*26*), inserted upstream to the SV40 early promoter (SVE) (devoid of the enhancer) are shown. The black box shows the first 250 base pairs of the CAT gene, and the line represents pUC13 DNA. Competition experiments were done as described (Fig. 2) with 0.6 μg of pSV2CAT as an indicator and 24 μg of the competitors. The values shown refer to percent of residual CAT activity determined after incubation of transfected cells in the presence of $5 \times 10^{-6} M$ $Cd^{2+}$.

hancers (*15, 31*). Since expression of SV40 depends on host factors, it is not surprising that the factors important for the expression of the virus have a normal role in the control of cellular gene expression, as has been shown for SP1 (*32*).


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23. Total cellular RNA (25 μg) was analyzed by primer extension with 5′–end labeled synthetic oligonucleotide primers complementary to positions +15 to +34 of the CAT gene (*20*) and +58 to +81 of the TK gene (*24*). The extended products were approximately 100 and 130 bases long, indicating correct initiation from the hMT-II$_A$ (*12*) and the SV40 early (*22*) promoters. The procedures used for primer extension analysis were as described by Walker *et al.* (*3*).
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26. The sequence of the synthetic MRE was 5′tcgaTGCGCCCGGCCCtcga, which is in agreement with a consensus of 14 such sites. Six of these sites have been tested directly for MRE activity (*6, 13*). The sequence also includes Sal I sticky ends used for cloning into pUC13. The sequence of the synthetic BLE is 5′ctagCGCGGGGCGTGTGCAGctag, in agreement with a consensus derived from seven MT genes. The sequence also includes the Xba I sticky end used for cloning into pUC13. The actual constructs used for competition contained fragments derived from the pUC13 vectors carrying the synthetic elements, which were inserted into the Bgl II site of the pUCAT2 vector (*15*) whose small Eco RI fragment (containing the majority of the CAT gene) has been deleted. Both the synthetic MRE and BLE were tested for activity in a transient expression assay and were found to be positive (A. Haslinger and M. Karin, unpublished results).
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# Activation of Smooth Muscle Contraction: Relation Between Myosin Phosphorylation and Stiffness


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**Contraction and myosin light-chain phosphorylation were measured in electrically stimulated tracheal smooth muscle. Latencies for the onset of force, stiffness, and light-chain phosphorylation were 500 milliseconds. Myosin light chain was phosphorylated from 0.04 to 0.80 mole of phosphate per mole of light chain with a pseudo–first-order rate of 1.1 per second with no evidence of an ordered or negatively cooperative process. Following the period of latency, stiffness increased with phosphorylation and both increased more rapidly than isometric force. The linear relation between stiffness and phosphorylation during activation suggests independent attachment of each myosin head upon phosphorylation.**


SMOOTH MUSCLE MYOSIN FILAMENTS are composed of myosin monomers that consist of two heavy chains [each with a molecular weight of 200,000 (200K)] and two each of two types of light chains (20K and 17K, respectively). Phosphorylation of the regulatory 20K light chains (P–light chain) of smooth muscle myosin by $Ca^{2+}$ and calmodulin-activated myosin light-chain kinase results in an increase in the actin-activated magnesium-dependent adenosinetriphosphatase ($Mg^{2+}$ ATPase) activity of myosin (*1, 2*). Conflicting biochemical data with smooth muscle myosin have been reported for two aspects of the activation process having important implications for regulation of contractile activity in the cell. (i) From kinetic studies, the mechanism of phosphorylation of the two heads of myosin in a filamentous form has been reported to conform to an ordered or negatively cooperative (*3, 4*), or an apparently random process (*5*). (ii) From studies on gizzard myosin, it has been concluded that both heads of myosin must be phosphorylated before the $Mg^{2+}$ ATPase activity of either head can be activated by actin (*3, 4*). Data with myosin from bovine stomach and swine pulmonary artery, however, allow the possibility that the $Mg^{2+}$ ATPase activity of each head is independently stimulated by phosphorylation of its P–light chain (*6*).

Contractile force in smooth muscle, as in skeletal muscle, is believed to result from the sliding of filaments due to the cyclic interaction of myosin with actin (*7*). In contrast to skeletal muscle, in which myosin P–light-chain phosphorylation modulates the contractility (*8*), P–light-chain phosphorylation in smooth muscle appears to be necessary for the contraction to occur (*2*). In addition, force development by a smooth muscle cell occurs only after a relatively long period of mechanical latency lasting some hundreds of milliseconds (*9*). Specific models relating mechanical activation to myosin P–light-chain phosphorylation in the smooth muscle cell cannot be tested in studies where cells in the tissue are slowly and asynchronously stimulated by agonist diffusing into the preparation or where values of phosphorylation vary only over a small range (*10*). We


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