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Genetics

Upstream promoter element of the human metallothionein-II_A gene can act like an enhancer element

(gene expression/transcriptional activator/deletion mapping/genetic duplication)

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ABSTRACT **Initiation of transcription by RNA polymerase II in eukaryotes is strongly increased by *cis*-acting genetic elements, known as activators or enhancers. Enhancers, first detected in simian virus 40 (SV40), were subsequently also found to control the expression of several cellular genes. The human metallothionein-II_A (*hMT-II_A*) gene, although inducible by heavy metals and glucocorticoids, is widely expressed in most cell types in the absence of inducers. Here we show that the high basal level of transcription of the *hMT-II_A* gene is due to the presence of an enhancer element within the *hMT-II_A* promoter region. The structural and functional organization of this cellular enhancer element in two direct repeats is strikingly similar to that of the enhancer element of SV40. This suggests a possible functional and evolutionary relationship between enhancers and upstream promoter elements.**

Transcriptional enhancers, first described in the genome of DNA viruses, activate transcription from neighboring promoters irrespective of orientation and distance (1–5). Later, this type of *cis*-activating genetic elements was also found to control the expression of cellular genes, usually in a tissue-specific manner (6–9). Some of the enhancers discovered consist of two direct repeats, each 60–80 base pairs (bp) long, while others do not exhibit such a structure. The presence of two repeats in some cases appears to be redundant, because a single copy is sufficient to activate transcription (1, 2). Yet, if this single copy is inactivated by mutation, its activity can sometimes be restored upon duplication of the defective copy (10, 11). Thus, the presence of repeats within enhancers seems to be a feature that is advantageous for achieving high levels of transcription, even though it may be dispensable in cases where a single element is already relatively efficient.

We are investigating the mechanisms that regulate the expression of the human metallothionein (MT) gene family (12), whose members are regulated differentially at the transcriptional level (13). Among them, the human MT-II_A (*hMT-II_A*) gene (12) is remarkable for its relatively high level of expression in most cell lines, even in the absence of inducers (unpublished results). Recently, we reported the identification of regulatory sequences involved in induction of the *hMT-II_A* gene by heavy metals and glucocorticoid hormone (14, 15). While analyzing various deletion mutants, we observed differences in the uninduced level of transcription, indicating the presence of an upstream promoter element, which is required for high transcriptional activity. Together with our former observation, that the *hMT-II_A* promoter is capable of activating the promoter of the herpes simplex thymidine kinase gene (*HSV-TK*) from a distance (14), it led us to the hypothesis that the 5′ flanking sequence of the *hMT-II_A* gene might also contain a constitutive enhancer element.

We present now a detailed analysis of this upstream promoter element and show that it acts like a bona fide enhancer element.

METHODS

Plasmid Constructions. All plasmid constructions were performed as described (14, 15). The 3′ deletion derivatives of the hMT-II_A 5′ flanking region were generated by BAL-31 digestion, with the deletion end point being designated $-n_1$. These hMT-II_A 3′ deletions were fused to the *HSV-TK* gene either at its 5′ end (MT/TK) or at its 3′ end (TK/MT) (seeFig. 1*A*). Two series of MT/TK plasmids were constructed by joining the hMT-II_A 3′ deletions to two different 5′ deletion mutants of the *HSV-TK* gene either at position $n_2 = -46$ or -109 derived from pTK_{-46} and pTK_{-109} (16). The single series of TK/MT plasmids contains the same hMT-II_A 3′ deletions inserted ≈300 bp 3′ to the intact *HSV-TK* gene derived from pTK_{-109}. These constructs contain hMT-II_A sequences starting at the *Hin*dIII site at position −770 and ending at various positions 3′ to it. pHSI is a 3′ deletion mutant, ending at position +69. pHSI-TK contains the *HSV-TK* gene fused with its *Bgl* II site (at +52) to the *Bam*HI site of pHSI. pHSIΔX-TK is a derivative of pHSI-TK in which the promoter sequence between the two *Xma* III sites (at −71 and −129) is excised and replaced with an *Xba* I linker. The parental plasmids for expression of the chloramphenicol acetyltransferase gene (*CAT*) were described (17, 18). pUCAT2 is a subclone of the simian virus 40 (SV40) promoter and the *CAT* gene from pA10CAT (18) into pUC8. It has a unique *Bgl* II site at the position −139 of the SV40 promoter and a unique *Bam*HI site at the 3′ end of the transcription unit. Both of these sites were used for insertion of fragments to be tested for enhancer activity. The fragments, named X and Y, contained *hMT-II_A* sequences from −67 to −132 and from −129 to −215, respectively.

Transfections and Analysis of Expression. Transfections into Rat 2 tk^- cells were done at either 0.5 or 1 μg of plasmid and 10 μg of calf thymus carrier DNA per plate (≈5×10^5 cells) using the Ca phosphate method as described (13). Transfections into HeLa cells were done at 20 μg of plasmid DNA as described (1–3). All the plasmids used for transfections were purified by double CsCl banding and reversed-phase chromatography on Elutip-d columns (Schleicher & Schuell), and their integrity and purity were verified by agarose gel electrophoresis and staining with ethidium bromide. Cells were harvested 48 hr after transfection with the CAT vectors, and the enzymatic activity of CAT was determined as described (17, 18). RNA for primer extension analysis was prepared by the guanidinium thiocyanate/CsCl method (19). Total cellular RNA (25 μg) was analyzed by

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Abbreviations: *hMT-II_A*, human metallothionein-II_A gene; bp, base pair(s); *HSV-TK*, herpes simplex virus thymidine kinase gene; *CAT*, chloramphenicol acetyltransferase gene; SV40, simian virus 40.

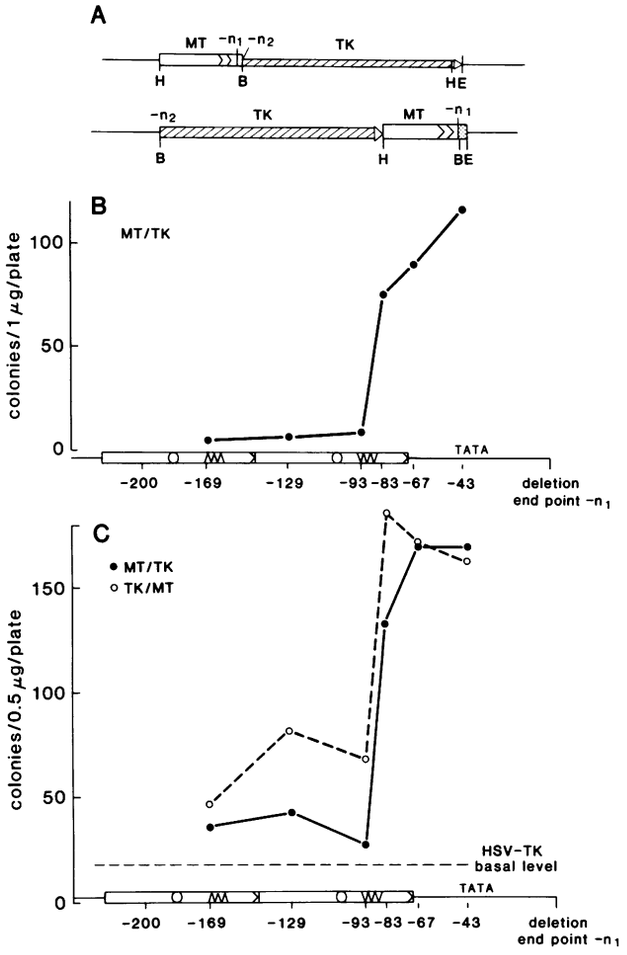

FIG. 1. Mapping the 3' boundary of the *hMT-II$_A$* activator. (*A*) Structure of the MT/TK and TK/MT plasmids. (*B* and *C*) The yield of tk$^+$ colonies after transfection of Rat 2 tk$^-$ cells with the various deletion mutants is plotted as a function of the deletion end point $-n_1$ within the hMT-II$_A$ regulatory region for (*B*) and the MT_{-n_1}/TK_{-46} and (*C*) the MT_{-n_1}/TK_{-109} (●) and TK_{-109}/MT_{-n_1} (○) constructions. The amount of DNA used was 1 μg per plate in *B* and 0.5 μg per plate in *C*. Numbers are averages from at least three plates.

using 5' end-labeled synthetic oligonucleotide primers as described (9).

RESULTS

Deletion Mapping of the Enhancer Element. In the first series of the MT/TK plasmids (Fig. 1*A*), several 3' deletion derivatives of the hMT-II$_A$ promoter were joined to a 5' deletion of the *HSV-TK* gene at position −46 (16). This generated hybrid promoters that contain proximal promoter elements of the *HSV-TK* gene and various upstream elements from the *hMT-II$_A$* gene. Some of these hybrid promoters still respond to induction with glucocorticoids or heavy metals (15). As shown in Fig. 1*B*, hMT-II$_A$ DNA fragments, which include the sequences from −770 to −83, increase the transfection frequency of the crippled *TK* gene ≈80-fold, indicating the presence within that region of an upstream element that can complement the *TK* gene deletion mutation. Deleting only 10 nucleotides further, from −83 to −93, leads to a sharp decrease in transfection efficiency, thus placing the 3' border of that upstream promoter element, required for high basal level of transcription, between nucleotides −83 and −93 of the *hMT-II$_A$* promoter. Analysis of TK mRNA expression in these cells by both blot hybridization and S1 nuclease mapping also indicated that this upstream element of the *hMT-II$_A$* gene potentiates accurate transcription from the deleted *TK* promoter manyfold (data not shown, but see figure 2 in ref. 15). Previously, we have mapped the 5' border of this element to be between nucleotides −93 to −74 in stably transfected Rat 2 cells (15) and between −160 and −96 in transiently transfected primate cells (unpublished data).

In the next series of MT/TK plasmids, the same 3' deletions of the hMT-II$_A$ promoter region were fused to the *HSV-TK* gene at position −109. The presence of the intact upstream promoter element of *TK* (16) in these plasmids is reflected in their overall higher transfection efficiency. Since these constructions now contain an intact *TK* promoter, the observed stimulation of transfection efficiency cannot be attributed to simple complementation of the crippled *TK* promoter. Yet again, deletion of the very same sequences between −83 and −93 causes a marked decrease in the enhancing activity of the hMT-II$_A$, confirming that the 3' end of the element required for the increased transformation frequency of the intact *TK* gene is also located between nucleotides −83 and −93 of the *hMT-II$_A$* promoter. The hMT-II$_A$ sequences had no effect on the start sites of TK mRNA, as analyzed by S1 nuclease mapping (data not shown).

To test the MT enhancer for its ability to function over a distance, we constructed the series of TK/MT plasmids, in which the 3' deletions were inserted downstream of the *TK* gene, separated from the *TK* promoter by ≈2.5 kilobases. As shown in Fig. 1*C*, the sequences within the *hMT-II$_A$* promoter can activate expression of the *TK* gene even over this distance, suggesting the presence of an enhancer element (1–5). Surprisingly enough, the same deletions that inactivate the function of the upstream promoter element also abolish the activity of the putative enhancer. The close correlation between the transfection efficiencies of the MT/TK and the TK/MT plasmids (Fig. 1 *B* and *C*) indicates that a sequence in the *hMT-II$_A$* promoter region that is essential for the activity of the upstream promoter element has (within 10 bp) the same 3' boundary as the sequence required for enhancer activity. We therefore conclude that both functions, if not identical altogether, must be encoded, at least in part, by the same region of DNA.

Since it appeared from both the 5' and 3' deletions that sequences between −83 and about −96 bp are essential for high basal transcriptional activity, we attempted to confirm these results by creating an internal deletion in this region of the *hMT-II$_A$* promoter. If the upstream element at this position were solely responsible for high promoter activity, its deletion should inactivate the promoter to an extent similar to the one observed with the 3' deletions. Therefore, we compared the transfection efficiencies of pHSI-TK, in which the intact *hMT-II$_A$* promoter is used to express the *HSV-TK* coding sequence lacking any *TK* promoter sequences with pHSIΔX-TK, which differs from pHSI only by an internal deletion between −71 and −129 bp. Surprisingly, the deletion of the upstream element from the *hMT-II$_A$* promoter did not have the expected negative effect. It only slightly reduced the transfection efficiency (Fig. 2), suggesting that this element might be present in more than only one copy; a situation that complicates the interpretation of simple deletion analysis (14).

Organization of the Upstream Element. The *hMT-II$_A$* gene sequence between positions −83 to −93, which defines the 3' end of this element, contains a sequence of 8 alternating purine–pyrimidine residues, which is perfectly repeated 80 bp further upstream between positions −162 and −169 (Fig. 3). Stretches of alternating purine–pyrimidines have the potential of assuming the left-handed Z-DNA structure under conditions of high underwinding (negative supercoiling), as shown by Nordheim and Rich (20) for such sequences within the SV40 enhancer. The same authors also noted the remark-

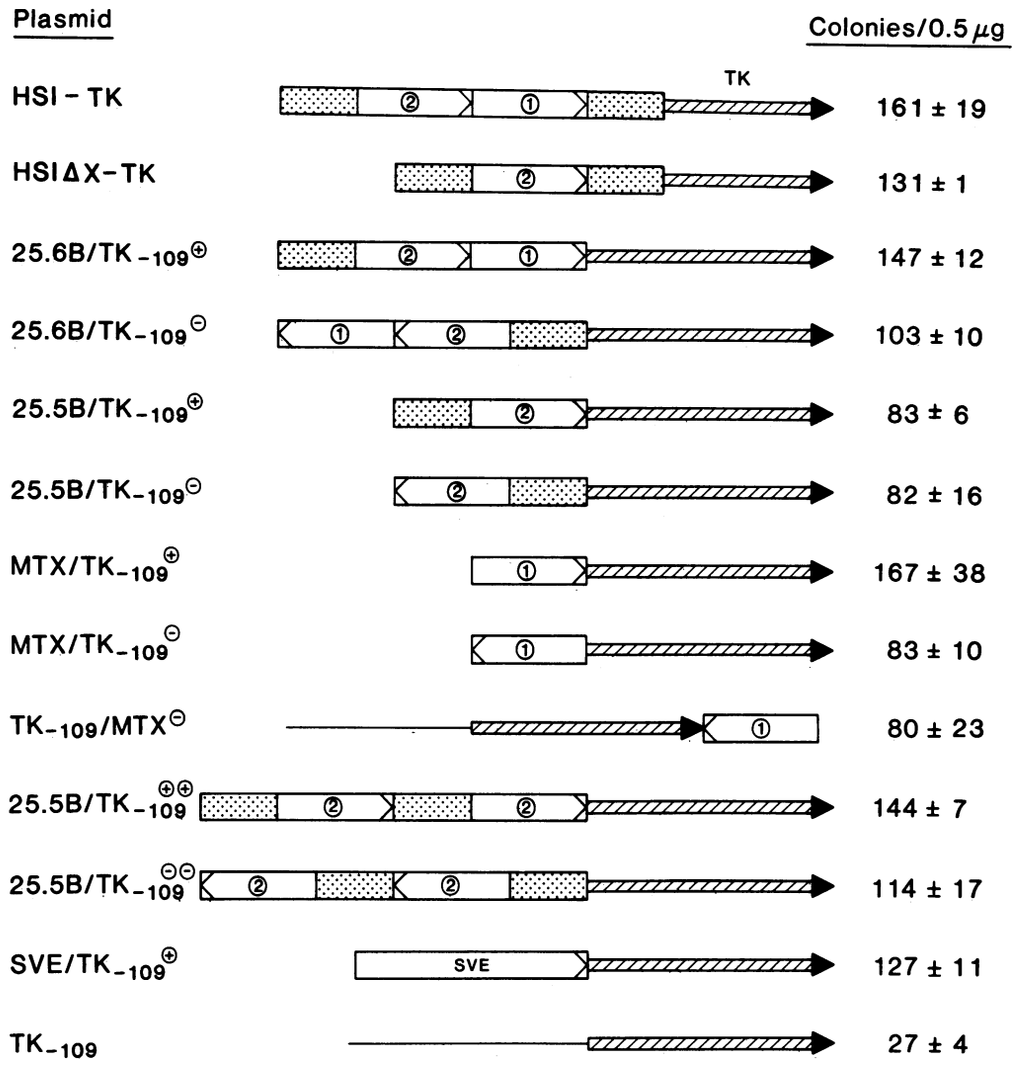

FIG. 2. Enhancer activity of the individual repeat units of the *hMT-II$_A$* activator. Various plasmids were constructed by standard recombinant DNA techniques to test the enhancer activity of hMT-II$_A$ 5′ flanking fragments. pHSI-TK contains hMT-II$_A$ sequences from −770 to +69 joined to the *TK* gene at position +52. pHSIΔX-TK is the same as pHSI-TK except for an internal deletion within the hMT-II$_A$ sequence (−71 to −129). 25.6B/TK$_{-109}$ contains hMT-II$_A$ DNA from position −67 to −338. 25.5B/TK$_{-109}$ contains hMT-II$_A$ DNA from position −129 to −338. This fragment is duplicated in 25.5B/TK$^{++}_{-109}$ and 25.5B/TK$^{--}_{-109}$. MTX/TK$_{-109}$ has hMT-II$_A$ sequences from positions −68 to −132. All these fragments were inserted with *Bam*HI linkers into the *Bam*HI site of pTK$_{-109}$ at position −109. SVE/TK$_{-109}$ has SV40 DNA from position 35 to 273 and contains the two SV40 repeats (kindly obtained from L. Laimins and G. Khoury). TK$_{-109}$/MTX$^-$ has hMT-II$_A$ sequences from −68 to −132 inserted ≈300 bp downstream of the *TK* gene. Numbers shown are averages (±SEM) of at least three plates each for each plasmid.

able recurrence of two such segments separated by a spacer of 60–80 bp in most viral enhancers (20).

Further close examination of the hMT-II$_A$ regulatory region reveals that it contains several more stretches of internal homology that can be summed up as two, somewhat divergent, direct repeats with a unit length of 70–80 bp (Fig. 3). Each of these repeats also includes a sequence with high homology to the consensus sequence of the adenovirus *E1A* gene enhancer (21). The proximal repeat also contains a sequence with some homology to the "core enhancer" sequence, which is common to a number of viral and cellular enhancers (22). Thus, overall arrangement of the hMT-II$_A$ repeats is very similar to that of the 72-bp repeats of SV40: both contain a common motif of one enhancer core sequence flanked by two segments of alternating purine–pyrimidine residues separated by 70–80 bp. However, the importance of this arrangement and, in particular, the possible involvement of Z-DNA in enhancer activity requires further investigation.

Enhancer Activity of the hMT-II$_A$ Repeats. To examine the functional significance of the two repeats within the *hMT-II$_A$* promoter region, we constructed a further series of plasmids. These constructs, described in Fig. 2, contain either both of the hMT-II$_A$ repeats, each of the isolated single repeats by itself, or a duplication of the distal repeat, which were inserted in both orientations either upstream or downstream to the intact *HSV-TK* gene. The effect of the hMT-II$_A$ derived fragments on expression from the HSV-TK promoter was determined by measuring their tk$^+$ transformation efficiencies after stable integration into the host genome. As shown in Fig. 2, the presence of two repeat units from the hMT-II$_A$ regulatory region stimulated the transfection efficiency of the *TK* gene, an effect shown earlier to be due to increased *TK* gene expression (23). There was no detectable difference between the activity of the two natural repeats (proximal and distal) or a duplication of the distal repeat.

To assay the enhancer activity also during transient expression, we constructed plasmids in which the expression of the *CAT* gene is controlled by the activity of an enhancer. The amount of CAT produced from such constructs can be used to quantitate the enhancer activity of various DNA sequences (18). We used as parental vector pUCAT2, which directs transcription of the *CAT* gene from the SV40 early promoter

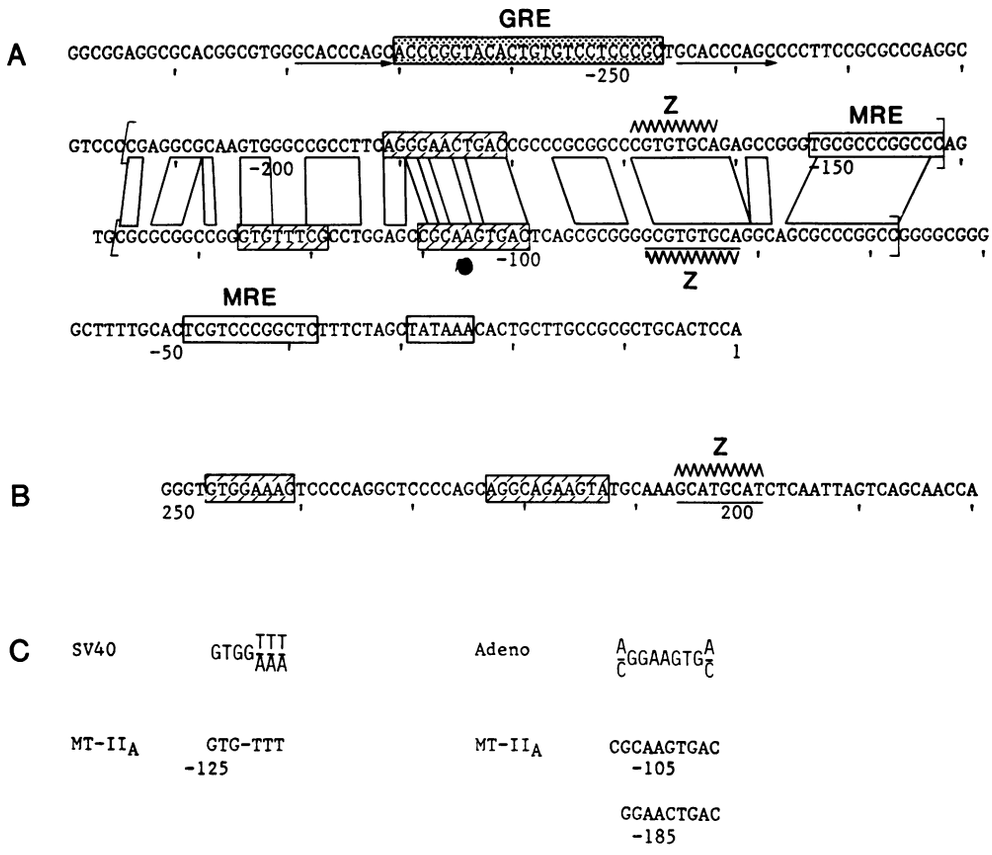

FIG. 3. The structure of the *hMT-II$_A$* activators. (*A*) The extent of homology between the two 70- to 80-bp repeats of the *hMT-II$_A$* is outlined by boxes, which encompass the nucleotide sequence identities. Transitions, although present, are not indicated. Also indicated are the metal responsive elements (MRE), the segments of potential Z-DNA, and two short repeats that flank the glucocorticoid responsive element (GRE). (*B*) The structure of the 72-bp repeat of the SV40 enhancer is shown for comparison to the *hMT-II$_A$* constitutive activator. The stretch of potential Z-DNA and the two different consensus sequences (20–22) are indicated. (*C*) Sequences within the *hMT-II$_A$* activator that are homologous to the "core" enhancer (22) and to the consensus sequence of adenovirus activators (21).

but lacks the SV40 enhancer. Into this plasmid we subcloned either the proximal repeat X (from −67 to −132) or the distal repeat Y (from −129 to −215) at −139 bp upstream of the transcription start site. Each repeat was inserted in both orientations either as single or double copy. The proximal repeat X was also inserted downstream of the transcription unit. The levels of CAT expressed from these plasmids during transient expression in HeLa cells are summarized in Table 1. As shown in Fig. 4*A*, two copies of either repeat were required to achieve significant enhancing activity in the transient expression assay. Primer extension analysis of RNA prepared from transfected cells confirmed that the presence of the repeats does not affect the selection of transcriptional start sites and that the CAT activities actually reflect differences in transcription levels of the *CAT* gene (Fig. 4*B*).

Table 1. Activities of CAT after transfection of HeLa cells with various plasmids

Plasmid	CAT activity, pmol of acetylchloramphenicol·hr^{-1} per mg of protein			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
pUCAT2	48	ND	206	81
pSV2CAT	2650	ND	3530	2410
pXCAT$^+$	90	ND	ND	ND
pXCAT^{--}	2910	ND	4370	1510
pCATX^{--}	610	ND	2060	1840
pYCAT$^-$	86	1020	ND	ND
pYCAT$^+$	110	1252	ND	ND
pYCAT^{++}	ND	2056	ND	ND

Each dish was transfected with 20 μg of DNA. Since transfection efficiencies depend on the exact growth conditions of the cells in each experiment, only values within each column can be compared for relative expression efficiency. ND, not determined.

DISCUSSION

The *hMT-II$_A$* gene is expressed in high levels in many cell types, including hepatic, renal, neuronal, lymphocytic, fibroblastic, and epithelial cell types (unpublished observations) and probably carries on important "housekeeping" functions (24). Our results show that the generally high expression of this gene is caused by an enhancer element located within its regulatory region. This enhancer includes an upstream promoter element around the −90 region (15), a position known to be occupied by upstream promoter elements of several other eukaryotic genes, including *TK* and globin (16, 25, 26). Yet, contrary to the upstream promoter element of *TK*, which only tolerates dislocations of <20 nucleotides (25), the upstream promoter element of *hMT-II$_A$* can increase transcription from heterologous promoters over a large distance (at least 2.5 kilobases) regardless of its orientation and position. Recently, Imperiale *et al.* (27) have demonstrated that the upstream promoter element of the adenovirus *E2* gene can also act as enhancer element. In this case, the sequences important for both functions were found between positions −21 and −79. The *hMT-II$_A$* enhancer is an example of an upstream promoter element of a cellular gene that can also function as an enhancer. However, it is likely, that other highly expressed housekeeping genes may contain similar elements.

Our findings that an upsteam promoter element can also act as an enhancer and that its biological activity is increased by duplication, together with those from Imperiale *et al.* (27), suggest that at least some enhancers might have evolved from upstream promoter elements by short genetic duplications. The differences between these two types of control elements could be quantitative rather than qualitative—for example, in their ability to transmit a signal over a distance or to function bidirectionally. Other data supporting this hypothesis are derived from the analysis of polyoma virus mutants capable of growth in teratocarcinoma stem cells (28, 29) and *cis*-acting

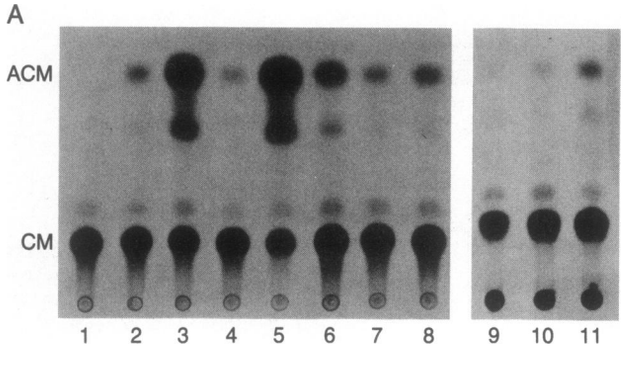

FIG. 4. Activity of the *hMT-II*$_A$ activator during transient expression. HeLa cells at a density of 5×10^5 cells per dish were transfected with 20 μg of plasmid DNA. After 48 hr the cells were harvested and RNA and cytoplasmic extract were prepared as described (17–19). (*A*) Assay for CAT activity after transfection with different plasmids. Lanes: 1, untransfected cells; 2, pUCAT2; 3, pSV2CAT; 4, pXCAT$^+$; 5, pXCAT^{--}; pCATX^{--}; 7, pYCAT$^-$; 8, pYCAT$^+$; 9, pYCAT$^+$; 10, pYCAT$^-$; 11, pYCAT^{++}. CM, chloramphenicol; ACM, chloramphenicol 3-acetate. (*B*) Mapping of the 5′ end of the CAT transcripts by primer extension. Total cellular RNA (25 μg) was hybridized to an end-labeled oligonucleotide primer complementary to nucleotides 15–34 of the coding sequence of the *CAT* gene and extended as described (9). Lanes: 1, primer without reverse transcriptase; 2, mock-transfected cells; 3–6, cells transfected with pSV2CAT, pUCAT2, pXCAT^{--}, and pCATX^{--}; M, *Hpa* II-digested pBR322. CS, correct start site.

activator-independent mutants of the *qa-2* gene of *Neurospora* (30). Many of these "promoter-up" mutations generate new enhancer elements that are associated with short duplications. Also, enhancerless SV40 mutants can give rise to SV40 variants by duplication of viral sequences that did not possess enhancer activity prior to the duplication (10, 11). Enhancer elements consisting of 60- to 81-bp repeats were recently found to regulate the activity of RNA polymerase I promoters as well (31). The sequence homology between the *Xenopus laevis* rDNA promoter and the 60- to 81-bp repeats indicates that they evolved from the promoter by short genetic duplications, thus supporting our hypothesis on the evolutionary and functional relationships between promoter and enhancer elements. Interestingly, most of these duplications fall in the same narrow size range of 60–80 bp. In this context, we would like to point out that this length is also close to the size of one nucleosomal turn (32). This could be a preferred distance for interaction between protein–DNA complexes required for gene activation (33). Whether the length of the repeats is an adventitious result of the mechanism by which these duplications arose or a necessary requirement for their function remains to be determined.

The presence of an enhancer element in the promoter of a human *MT* gene may also be important for the development of cases of acute myelomonocytic leukemia with chromosomal rearrangements involving the cluster of *MT* genes on chromosome 16 (34) by transcriptional activation of a putative oncogene present on the short arm of the chromosome.

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