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Gene activation properties of a mouse DNA sequence isolated by expression selection

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ABSTRACT

The MES-1 element was previously isolated from restricted total mouse cellular DNA by "expression selection" - the ability to reactivate expression of a test gene devoid of its 5' enhancer sequences (1). Mes-1 has been tested in long-term transformation and short-term CAT expression assays. In both assays MES-1 is active independent of orientation and at a distance when placed 5' to the test gene. The element is active with heterologous promoters and functions efficiently in both rat and mouse cells. MES-1 activates expression by increasing transcription from the test gene's own start (cap) site. Thus the expression selection technique can be used for the isolation of DNA sequences with enhancer-like properties from total cellular DNA.

INTRODUCTION

Transcriptional enhancers are small sequence elements which can function from a distance in an orientation-independent fashion to increase the expression of a gene via initiation of transcription from the gene's own start (cap) site. Although enhancer elements were initially identified in viruses (2-4), more recently a number of sequence elements with some of the properties of enhancers have been found associated with cloned cellular genes (for reviews see 5,6). We have taken an alternative approach called "expression selection" for the isolation of cellular enhancer sequences (1,7). This relies on the ability of restriction fragments of genomic DNA to confer expression upon a selectable gene devoid of its own enhancer element. A 149 bp fragment of mouse cell DNA formerly called H-2 and henceforth referred to as MES-1 (mouse expression sequence 1) was isolated using this expression selection technique (1). In this report the ability of MES-1 to enhance the expression of heterologous genes lacking an enhancer element has been characterized using long-term and short-term expression assays. MES-1 has been found to share a number of properties with classical enhancer elements.

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MATERIALS AND METHODS

Plasmids

All plasmids contain the ampicillin resistance gene of pBR322 and were propagated in E.coli strain DH-1 (8). The supercoiled plasmids used for DNA transfection were purified twice by centrifugation, first by density in CsCl and second by velocity sedimentation through sucrose.

Expression Assays

RAT-1 and Ltk(-) cells were used for short-term CAT assays. Cells were plated the day before transfection onto 5 cm petri dishes containing 2×10^5 cells in Eagles medium containing 10% fetal calf serum. The medium was changed 4 hours prior to transfection. DNA transfections and assay of CAT activity were carried out essentially as described by Gorman *et al*. (9). Plasmid DNA was precipitated at a concentration of 20 μg/ml in the transfection mix. Ten μg of plasmid DNA were transfected onto each petri dish containing a total volume of 5 ml medium. Cells were incubated with the precipitate for 12 hours at 32°C, washed twice with versene and provided with fresh medium. Cells were then incubated for 48 hours at 37°C after which time the cells were harvested and assayed for CAT activity. The results of CAT reactions were visualised by autoradiography of the TLC plates and the reaction products thus localized were extracted and quantified by scintillation counting (9). The long-term transformation assays using Rat-1 cells were performed as described by Fried *et al*. (1). The cells were stained with Leishman's stain seventeen days after transfection and the number of densely packed transformed foci counted.

Transient Expression/S1 Nuclease Protection Assays

Ten ml of calcium phosphate precipitate containing 15 μg/ml supercoiled plasmid was transfected onto 5×10^6 Rat-1 cells on a bioassay tray containing 90 ml Eagles medium supplemented with 10% fetal calf serum. After incubation at 37°C for 5 hours, the precipitate was removed by washing with versene and the cells were refed with fresh medium. Forty hours later total cellular RNA was prepared by lysis with guanidinium isothiocyanate as described (8). An AccI digest of pPy70 was dephosphorylated using calf intestinal alkaline phosphatase (Boehringer) and the 5' ends labeled using γ-^{32}P ATP (3000 Ci/mmol, Amersham) and polynucleotide kinase (Boehringer). After digestion with BamHI, the 401 bp double stranded BamHI-AccI fragment spanning Py nts. 5265-371 was isolated by agarose gel electrophoresis and used as a probe. Forty μg of total cellular RNA

was annealed with 40 000 dpm of probe in 20 µl of hybridisation buffer as described (10). S1 digestion was performed for 2 hours at 14°C with 500 units/ml S1 nuclease (Sigma) and resistant fragments were separated on a 5% polyacrylamide/7M urea sequencing-type gel.

RESULTS

Vectors Used for Expression Assays

The 149 bp MES-1 sequence was originally isolated as a BamHI-HaeII restriction fragment (1). The HaeII cleavage site was changed to a BglII restriction site by linker addition in order to facilitate easy insertion, in both orientations, of the fragment into the vectors used for the long- and short-term expression assays. These vectors are shown in Fig. 1.

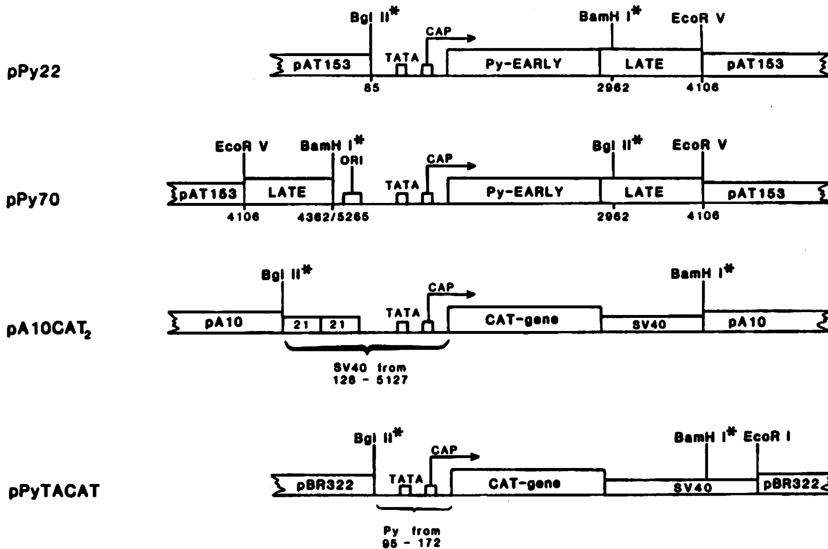


Fig. 1. Expression vectors. The relevant regions of the plasmids pPy22 and pPy70 (long-term transformation vectors) and pA10CAT2 and pPyTACAT (short-term CAT expression vectors) described in the text are shown. The BamHI and BglII restriction sites used for insertion of the MES-1 element are indicated by asterisks (*) and the direction of transcription of the respective test genes by arrows. The plasmid parts of the respective vectors (pAT153, pA10, pBR322), the Py origin of DNA replication (ORI), the Py or SV40 TATA box sequences (TATA) and cap sites (CAP) and the other Py and CAT sequences are indicated. The parts of the CAT vectors labelled SV40 3' to the CAT gene contain the SV40 early region splice and poly-adenylation signal. Numbering under the pPy22 and pPy70 vectors refers to Py nucleotide numbers.

The pPy70 vector used for the long-term transformation assays contains polyoma virus (Py) DNA linearised at the EcoRV site and lacking 633 bp of Py sequence between the BamHI site at nt 4632 and the PvuII site at nt 5265. The PvuII site at nt 5265 has been converted to a BamHI site by linker addition. The pPy22 long-term transformation vector contains 4.0 kb of Py sequence, between the NarI site at Py nt 85 and the EcoRV site at Py nt 4106, which has been cloned into the pAT153 plasmid between the EcoRV site at nt 185 and the NarI site at nt 1204. The single NarI site in pPy22 has been converted to a BglII site by linker addition. The Py sequences in both vectors contain the complete Py early region (transforming region) with its associated TATA box and cap site and part of the Py late region (11), (pPy70 also contains the origin of viral DNA replication), but lack the Py enhancer region (nts. 5021-5265) (12) and are thus greatly inhibited in their transforming ability (1,7). For insertion of the BamHI-BglII MES-1 fragment 5' to the Py transforming gene the BamHI site at Py nt 5265 in pPy70 or the BglII site at Py nt 85 in pPy22 was used. These sites are about 65 bp (pPy22) and 180 bp (pPy70) upstream of the major sites used for the initiation of transcription of the Py early mRNAs (cap sites) (13). For insertion of the MES-1 fragment 3' to the transforming region the HincII site at Py nt 2962, just downstream of the Py early region, was used after conversion to a BglII site in pPy70 or to a BamHI site in pPy22 by linker addition.

The plasmids pA10CAT2 (14) and pPyTACAT were used for the short-term assays (Fig. 1). The pA10CAT2 plasmid contains the bacterial chloramphenicol acetyl transferase (CAT) gene which is preceded by the SV40 promoter element containing the 21 bp repeats and followed by the SV40 early region splice and polyadenylation signals. The enhancerless pA10CAT2 plasmid is greatly inhibited in inducing the production of CAT activity after transfection into mammalian cells (14). The BamHI-BglII MES-1 fragment was inserted 5' to the CAT gene at the BglII cleavage site, at the join of the SV40 promoter element with the plasmid sequences, which is about 140 bp upstream from the major cap sites of the SV40 early region. In the pPyTACAT vector a Py fragment containing the Py early region TATA box and cap sites has been inserted 5' to the CAT gene of the pSV0CAT plasmid of Gorman et al. (9). The HaeII site at Py nt 95 and the BstXI site at Py nt 166 at the ends of this Py fragment were converted into BglII and BamHI sites respectively by linker addition and inserted into the BglII site (converted from the original HindIII site) of pSV0CAT so

Table 1 **Effect of the MES-1 fragment on expression of long-term transformation vectors**

Plasmid	transformed foci per 100 ng of DNA
pPy22	3
→ pPy22	184
← pPy22	37
pPy22 →	5
pPy22 ←	4
pPy70	3
→ pPy70	172
← pPy70	191
pPy70 →	3
pPy70 ←	7
pPy	192

The arrows indicate the orientation of the MES-1 element when placed either 5' (before) or 3' (after) in either the pPy70 or pPy22 vectors. The arrow pointing from left to right represents the orientation in which MES-1 was originally isolated. pPy is a plasmid that contains the Py early region and its associated enhancer element.

that the BglII site in front of the Py fragment was maintained (Fig. 1). The MES-1 fragment was inserted 5' to the CAT gene at this BglII site which is about 55 bp upstream from the Py early region major cap sites. In both pA10CAT2 and pPyTACAT the MES-1 fragment was inserted 3' to the CAT gene at the BamHI site just 3' to the SV40 polyadenylation signal (Fig. 1).

MES-1 Expression Activity

The results of the induction of transforming activity by MES-1 when placed in either orientation either 5' or 3' to the Py transforming region in pPy70 and pPy22 are presented in Table 1. When placed in the 5' position in pPy70, MES-1 induces an orientation-independent increase in transforming activity of approximately 60 fold. No increase in activity

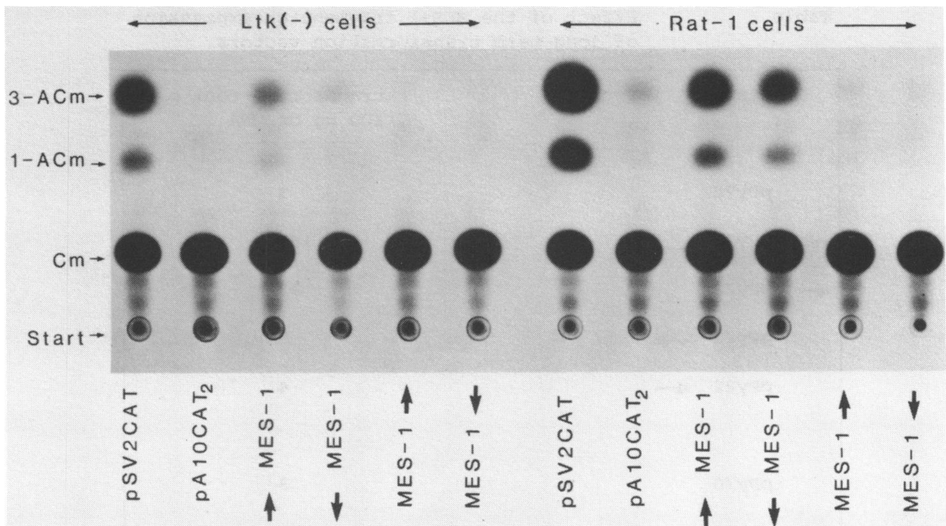


Fig. 2. CAT assay using the pA10CAT2 vector. The MES-1 sequence was inserted into pA10CAT2 either at the 5' or 3' position to the CAT gene (see Fig 1) and assayed after transfection into mouse Ltk(-) or Rat-1 cells. The pSV2CAT plasmid containing the SV40 enhancer (9) and the enhancerless pA10Cat2 (14) served as positive and negative controls respectively. An autoradiogram of the reaction products is shown after fractionation on a TLC plate. The orientation of MES-1 is indicated by the arrows with the arrow going from left to right indicating the orientation of MES-1 as originally isolated (1). Cm = Chloramphenicol, 1-ACm, 3-ACm = 1- or 3- Acetylchloramphenicol.

was detected when MES-1 was inserted in pPy70 in either orientation at a single position 3' to the transforming region. The MES-1 fragment also increased transforming activity in an orientation-independent manner when placed in a 5' position in the pPy22 vector, but the increase in activity in one orientation was always 3-10 fold greater than that in the other orientation.

The MES-1 fragment also induced an increase in CAT activity in the short-term expression assays in both rat and mouse cells (Fig. 2 and Table 2). Whereas there was only a 2-5 fold difference in increased activity between the two orientations with pA10CAT2, the increase in activity in pPyTACAT was 7 to 50 fold higher in one orientation than in the other (Table 2). The orientation showing the greater activity in pPyTACAT was the same orientation that showed an increased activity with pPy22 in the long-term expression assays (Table 1). Little or no increase in CAT act-

Table 2   Enhancement of CAT gene expression by the MES-1 sequence

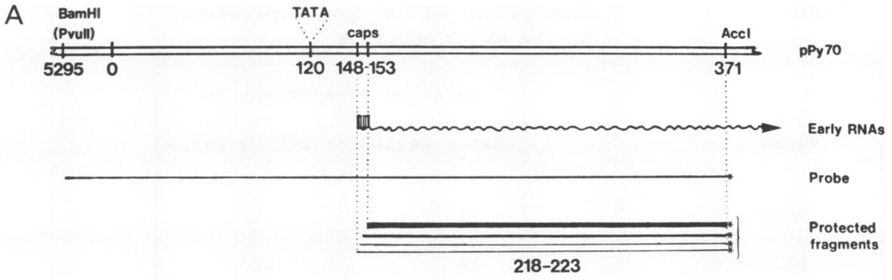
Plasmid	relative enhancement in Rat-1 cells	relative enhancement in Ltk(-) cells
pA10CAT2	1	1
→ pA10CAT2	12	22
← pA10CAT2	9	9
pA10CAT2 →	1	1
pA10CAT2 ←	1	1
pPyTACAT	1	1
→ pPyTACAT	33	98
→ pPyTACAT	5	2
pPyTACAT →	1	1
pPyTACAT →	1	1
pSV2CAT	45	43

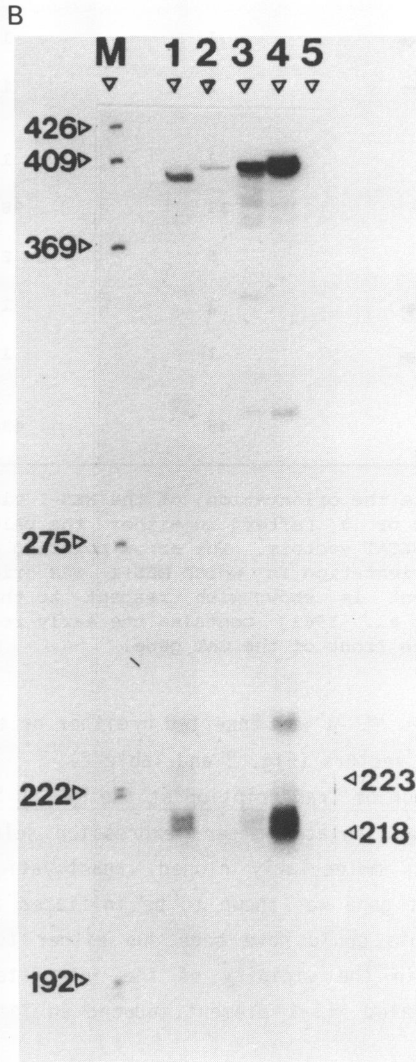
The arrows indicate the orientation of the MES-1 element when placed either 5' (before) or 3' (after) in either the pA10CAT2 (Laimins et al. 1984) or pPyTACAT vectors. The arrow pointing from left to right represents the orientation in which MES-1 was originally isolated. Relative enhancement is shown with respect to the vectors alone. pSV2CAT (Gorman et al. 1982) contains the early region promoter and enhancer of SV40 in front of the CAT gene.

ivity was detected when MES-1 was inserted in either orientation at a single 3' position in the two vectors (Fig. 2 and Table 2).

MES-1 Induces Initiation of Transcription at the Py Cap Site

In the cell line, isolated after "expression selection," from which the MES-1 element was molecularly cloned, reactivation of transcription of the selectable test gene was shown to be initiated at the correct start (cap) sites (1). This could have been due either to the MES-1 element or another sequence in the vicinity of the integrated test gene. The finding that the isolated MES-1 element induced an increase in biological


A
BamHI
(PvuII)
TATA
caps
AccI
pPy70
5295
0
120
148-153
371
Early RNAs
Probe
Protected fragments
218-223


B
M
1
2
3
4
5
426
409
369
275
222
192
223
218

activity of a test gene, in an orientation-independent manner, when positioned at different distances 5' to the test gene (see above) indicated that MES-1 contains gene enhancer activity. Thus it would be expected that the MES-1-induced increase in biological activity would be due to an increase in transcription and that this increased transcription would be initiated at the test gene's cap site.

The positions and relative abundance of the 5' ends of transcripts induced by MES-1 were determined after transient expression in rat cells. Transcripts obtained by insertion of MES-1 into the pPy70 vector in both orientations are compared with those from the vector alone and from a similar plasmid containing the Py early region and Py enhancer (Fig. 3). The major cap sites for transcription of the Py early region show microheterogeneity and have been mapped between Py nts 148-153 (13). In both orientations MES-1 induced a substantial increase in transcription initiated from the bona fide Py cap sites (Py nts 148-153) compared to the vector alone. A larger amount of transcription was consistently observed in the transient assay when MES-1 was in one orientation (opposite to that as originally isolated) in the pPy70 vector (Fig. 3). In the long term transformation assay no significant differences were observed between the two orientations of MES-1 in the pPy70 vector (Table 1). This apparent discrepancy may reflect the different natures of the two assays. The transient assay measures the average transcriptional activity of a substantial number of cells soon after transfection. In contrast in the long-term assay a stably transformed phenotype is monitored in a small fraction of the cells weeks after transfection. Attempts to compare the two assays may consequently lead to artificial contradictions.

Fig. 3. MES-1-induced transcription from the Py early cap sites. (A) The transcription initiation region of the pPy70 vector aligned with the major early capped Py RNAs, initiated at nts.148-153 (13). The 401 bp fragment between Py nts. 5265 and 371 was used as a probe in an S1 nuclease mapping experiment after 5' end labelling at nt.371. Py RNAs using the bona fide Py cap sites protect fragments in the range 218-223 nucleotides. (B) S1-resistant fragments produced by annealing the probe with RNAs transiently expressed from (1) a plasmid pAT153 clone containing a BamHI-linearised Py molecule (contains Py enhancer). (2) The pPy70 vector lacking the Py enhancer. (3) pPy70 containing the 149 bp MES-1 sequence inserted at the pPy70 BamHI site with the MES-1 fragment in the orientation as originally isolated (1). (4) pPy70 containing the 149 bp sequence in the opposite orientation. (5) The probe annealed to yeast tRNA. Size markers (M) are end-labelled DdeI fragments of the pAT153 plasmid containing the BamHI-Linearised Py molecule.

DISCUSSION

The isolation of the 149 bp MES-1 element from mouse genomic DNA was facilitated by the element's ability to reactivate a selectable gene devoid of its 5' expression sequences (1). The results presented here show that MES-1 has many of the properties associated with gene enhancer elements. MES-1 can activate a test gene in an orientation-independent manner when placed at least 180 bp upstream from the cap site of the test gene. The MES-1-induced increase in biological activity is the result of an increase in transcription and the MES-1-induced transcripts are primarily initiated at the test gene's own cap site. MES-1 can activate heterologous (Py and SV40) "promoters" in both rat and mouse cells. In addition MES-1 has also been shown to be active in dog, monkey and human cells (V. von Hoyningen-Huene unpublished results). The MES-1 element does not appear to contain any of the consensus sequences associated with other classes of enhancer elements (1) nor does it appear to contain the consensus sequence for the Spl factor binding sites (15).

The activity of MES-1 appears to be sensitive to sequences in the vicinity of its location. Whereas MES-1 was not very active in one orientation when placed 55 bp (pPyTACAT) or 65 bp (pPy22) from the Py cap site it was highly active in the same orientation when placed 180 bp (pPy70) from the Py cap site or 140 bp in front of the SV40 cap site in pA10CAt (Fig. 2, Tables 1 and 2). This sensitivity to location may explain why MES-1 has not been found to be active in the two positions tested at a 3' position to a test gene. Alternatively, MES-1 may represent a class of cellular enhancer-like sequences which have only some of the classical gene enhancer properties defined initially in viruses. The activities of enhancer-like sequences associated with the human c-fos gene (16), the rat insulin I gene (17) and the herpes simplex virus intermediate early gene 3 (18, 19) have also been found to be sensitive to position and orientation. For instance the 5' regulatory sequences of the c-fos gene are active in an orientation-independent manner only at a limited distance 5' to the c-fos gene and have little or no activity when placed 3' to the c-fos gene (16).

Recent results indicate that a given sequence can have both enhancer and promoter activities (20, 21). The promoter sequences associated with the Py early region have not yet been defined. Thus it is possible that in at least some of the Py constructs MES-1 is displaying promoter-like activity. Although some promoter elements can be active independent of

orientation they do not appear to be active when moved away from the test gene (22,23). The MES-1 element is active both close to and at a distance from the test gene in the Py constructs (Tables 1 and 2). In the case of the pA10CAT2 plasmid which contains the SV40 21 bp repeat promoter element MES-1 induces an orientation-independent increase in CAT activity, when placed in front of the SV40 promoter (Fig. 2 and Table 2), as would be expected for an enhancer sequence. The enhancer/promoter activity of MES-1 in its natural chromosomal location in the mouse genome awaits further elucidation.

The "expression selection" technique was developed in order to be able to isolate expression sequences independent of their association with a cloned gene (1,7). The "enhancer trap" method of Weber et al. (24) was also devised in order to select and isolate enhancer elements. The "enhancer trap" method relies on intracellular recombination between transfected enhancer sequences and an enhancerless SV40 vector to produce an infectious SV40 molecule. There is a constraint on the size of the DNA incorporated in the vector as the hybrid molecule must be packaged into SV40 virions. The incorporated enhancer must be able precisely to regulate the SV40 early and late region transcription so that the hybrid viral DNA can efficiently replicate and be packaged into new infectious virus particles during the lytic cycle in monkey cells. Using this method the enhancer sequences once covalently linked to the SV40 DNA are easily isolated from the abundant replicated extrachromosomal circular SV40 DNA molecules (24).

In the "expression selection" method the enhancer sequence and the test gene are recombined (ligated) in vitro. The hybrid DNA molecules are then transfected into cells and those expressing the test gene from a chromosomal (integrated) location are selected and analysed. There are no size constraints on the DNA that contains the enhancer element. All that is required of the enhancer element is that it restores activity to the test gene in its chromosomal location. In contrast the "enhancer trap" requires that a number of temporal events be precisely regulated by the enhancer to produce infectious SV40 virus. The isolation of enhancer like sequences detected by "expression selection" requires laborious cloning from cellular DNA (1,7) as opposed to the easy isolation of such sequences from abundant extrachromosomal circular DNA (see above) using the "enhancer trap" method. Although the "enhancer trap" method has proven very useful in detecting enhancer elements when large amounts of

a cloned DNA, especially from viruses, are transfected with the enhancerless SV40 vector (20, 24-28), this method has not been successful in selecting enhancer elements from total cellular DNA (20, 24). In contrast the "expression selection" method has been successful in selecting sequences with enhancer properties from total cellular DNA (1,7) possibly as a result of the less stringent requirements for gene activation.

Once isolated an enhancer-like sequence can be used as a probe for the cloning of surrounding DNA sequences which may contain associated "companion" genes. The finding that MES-1 is located between the 5' ends of two divergent transcription units (T. Williams and M. Fried manuscript submitted) is consistent with the enhancer-like activity of the MES-1 sequence presented here. Furthermore a second sequence isolated by "expression selection" by Ford et al. (7) also appears to contain a gene enhancer sequence. Thus the use of "expression selection" appears to be a fruitful technique for the isolation of cellular expression sequences with enhancer activity.

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