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Transcription from a plant gene promoter in animal cells

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ABSTRACT

The promoter segment of a plant gene (maize alcohol dehydrogenase 1 (Adh1)) has been fused to two bacterial reporter genes, Ecogpt (1) and neo (2), in pSV2-derived vectors and introduced into cultured mammalian cells by DNA transfection. The pAdh1-gpt plasmids transformed the recipient cells for resistance to mycophenolic acid plus xanthine (3) and the analogous pAdh1-neo plasmid transformed cells to G418 resistance (2). S_1 analysis of transient transfections of CV1 cells with various derivatives of pAdh1-gpt confirmed that production of gpt mRNA is initiated at the Adh1 promoter at and near the same site used in transcription of the intact Adh1 gene in maize. Moreover, expression of the Adh1 promoter was increased 10-20 fold if the SV40 early region enhancer sequence was included in the same molecule.

INTRODUCTION

There is a marked resemblance between the arrangement of functional elements of a number of plant and animal genes. For example, the plant genes leghemoglobin (4) and phaseolin (5) contain TATA or Goldberg-Hogness boxes (6), 20-30 bp upstream from the CAP site and sequences at the intron/exon junctions are similar. Two alcohol dehydrogenase genes have been cloned from maize and their nucleotide sequences determined (7, 8). The Adh1 gene contains a sequence (TATATAAT) that resembles a TATA box of a typical animal gene, 37 bp upstream of the site of transcription initiation; there is also a GGCCAAACC sequence that may correspond to the CCAAT box at position -90 bp. A comparison of the cDNA and genomic sequences revealed the existence of 9 introns in the Adh1 gene, each with junction sequences that conform to the consensus sequence at the splice sites of animal gene introns. Although the Adh1 mRNA is polyadenylated, there was no signal resembling AATAAA in the cDNA clone in a position similar to that of the consensus sequence in animals.

The functional significance of plant gene sequences can be tested by

introducing them into cells where they are expressed. Lacking a homologous cell culture system for monocotyledonous plants such as maize, we investigated the utilization of the Adh1 gene promoter in mammalian cells. This was accomplished by fusing the Adh1 gene promoter to the Ecogpt (1) or neo (2) markers in pSV2-like transducing plasmids and measuring the expression of gpt or neo after introduction into CV-1 and COS monkey cell lines. Our results indicate that the Adh1 promoter can transcribe either marker sufficiently to yield stable gpt or neo transformants. Furthermore, the gpt mRNA transcribed from the Adh1 promoter during transient transfection of monkey cells is initiated at or near the RNA initiation site used in maize. The formation of the XGPRT enzyme in transformed and transiently transfected cells also indicates that the fusion mRNA is translated. The Adh1 promoter was only about 2 percent as active as the SV40 early region promoter in expression of gpt, but Adh1 promoter-mediated expression was increased 10-20 fold if the SV40 enhancer region was included in the same molecule and several-fold in COS cells if the SV40 origin was present to permit amplification of the vector.

MATERIALS AND METHODS

Animal Cells

Monkey cells (COS and CV1) were grown in Dulbecco modified minimal medium supplemented with 5% newborn calf serum, penicillin and streptomycin.

Enzymes and Reagents

Restriction endonucleases EcoR1 and BamH1 were gifts from P. Modrich (Duke University) and C. Mann (Stanford University) respectively. Other restriction enzymes were purchased from New England Biolabs. E. coli DNA polymerase 1 and T4 ligase were gifts from S. Scherer (Caltech). Calf alkaline phosphatase and S1 nuclease were purchased from Boehringer Mannheim. The antibiotic G418 was generously provided by Dr. P.J.L. Daniels of Schering-Plough Corporation. Stock solutions contained 4 mg/ml G418 in 100 mM HEPES (N-2 hydroxy-ethyl piperazine N'-2' ethane sulphonic acid) buffer, pH 7.3. The amount of G418 used takes account of the fact that the G418 was only 50% pure.

Construction of Recombinant Molecules

a. Isolation of the maize Adh1 promoter. The sequence of the -160 to +200 region of the maize Adh 1 gene is shown in Fig. 1a. The previously identified RNA transcription initiation site, the TATAA box and the CCAAT

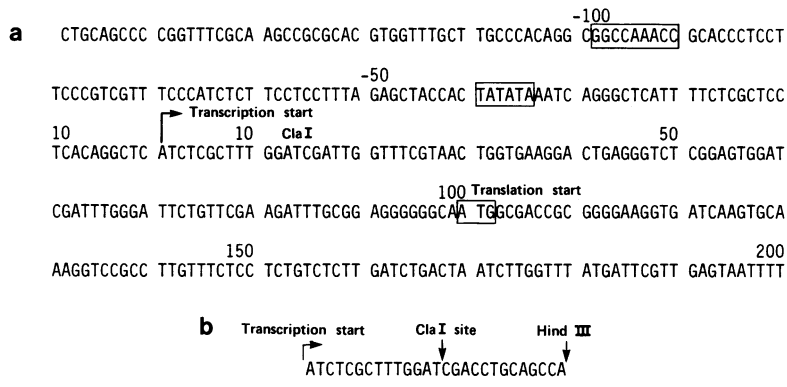

Fig. 1. a) Sequence of the Adh1S gene from maize with relevant features marked. The ClaI site used in the isolation of the promoter sequence is indicated and the newly generated sequence from the transcription start site to the HindIII site of the Adh1S promotor is shown (b).

box region are marked. The fragment extending from a BamH1 restriction site, 1.1 kb upstream (-1100 bp) to a ClaI site at +14 (a nucleotide between the transcription and translation start points) was excised and ligated between the BamH1 and AccI restriction sites of the pUC 8 plasmid (9). After cloning this segment was removed by successive BamH1 and HindIII endonuclease digestions. This yields a 1.1 kb fragment, flanked by BamH1 and HindIII cohesive ends, containing the probable promoter sequence of the Adh 1 gene (Fig. 1b).

b. Plasmids with the maize Adh1 promoter fused to bacterial genes. The ability of the Adh1 promoter to initiate transcription in mammalian cells was tested by fusing it to reporter genes, either Ecogpt (1) or neo (2), whose expression can be readily detected or selected for in transfected cells (2,3). This experimental design is occasionally confounded by spurious transcription initiations within pBR322 DNA sequences that are 5' of the promoter being tested. This complication can be minimized by the insertion of one or more (T. Kadesch, personal communication) copies of the SV40 early region polyadenylation signal upstream of the Adh1 promoter segment (Fig. 2). As a result of this modification, the expression of the marker genes should accurately reflect the activity of the Adh1 promoter. To construct the pSV2A type plasmids (i.e. those containing two polyadenylation signals upstream of the promoter site), the PvuII restriction site on the upstream edge of the SV40 ori segment was converted to a BamH1 restriction site. The desired fusion plasmids pAdh1S2A-gpt and

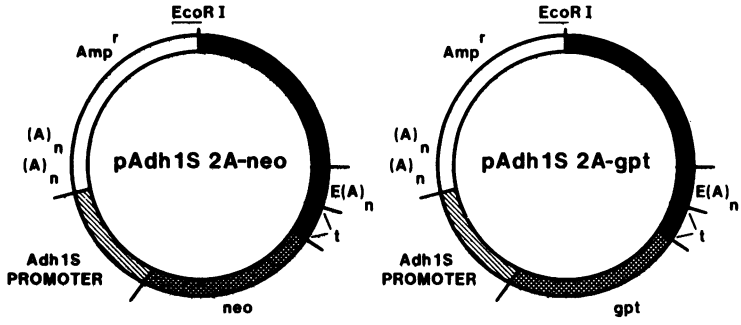

Fig. 2. Structure of the hybrid plasmids pAdh1S2A-neo and pAdh1S2A-gpt. The plasmids are composed of DNA segments from various sources: pBR322 represented by the clear arc, contains the pBR322 origin of replication (pBR322 ori) and the β lactamase gene (Amp^r); the cross hatched segment contains the selectable gene - either the neo gene (1.4 kb) fragment from Tn5 (2) or gpt (1). The SV40 sequences are shown in solid black and contain the small tumour - antigen intervening sequences (0.45-0.44 map units) and the sequence at which termination and polyadenylation of SV40 early transcripts (0.19-0.1 map units) occurs.

pAdh1S2A-neo were constructed by appropriate HindIII and BamHI cleavages and replacement of the SV40-derived promoter with the Adh1 promoter segment (Fig. 2).

To permit the plasmids described above to be amplified in CV-1 cells the SV40 ori sequence and the region encoding the large T antigen genes were introduced into the plasmid (pAdh1S2A-gpt-SVT, Fig. 3a). To test for expression of Adh1S2A-gpt in COS cells, which produce large T antigen constitutively (10), the plasmid was also modified to contain only the SV40 ori sequence; the SV40 EcoRII-G fragment, which contains the SV40 ori sequence (11), was suitably modified, cloned to have EcoRI cohesive ends and then inserted at the single EcoRI restriction site of pAdh1S2A-gpt (pAdh1S2A-gpt-ori, Fig. 3b). Another derivative of the pAdh1S2A-gpt series contained the enhancer segment of the SV40 early region promoter; this was provided by the SV40 72 bp repeat sequence (12) inserted at the BamH1 restriction site beyond the polyA site of the gpt transcription unit (pAdh1S2A-gpt-EH, Figure 3c).

DNA transfection and selection of transformed cells.

Approximately 5×10^6 cells per 10 cm dish were transfected with supercoiled plasmid DNA (10 μg., without carrier) using the calcium phosphate precipitation technique (13) and glycerol shock after 4 hrs (14). Transformants, resistant to G418, were recovered as previously described

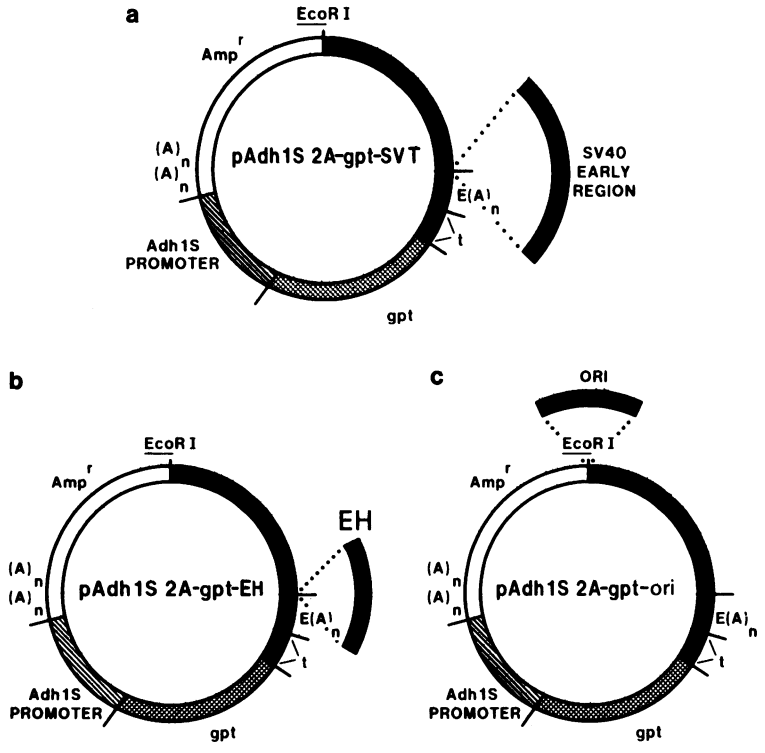

Fig. 3. Structure of the pAdh1S2A-gpt plasmids containing additional SV40 derived sequences. a) pAdh1S2A-gpt-SVT contains an insertion of the SV40 early region in the Bam site of pAdh1S2A-gpt. b) EH refers to the enhancer sequence (72 bp large repeat) from SV40 (13). c) ori refers to the origin of replication of SV40 in the form of the R11G fragment of SV40 (12).

(2) and cells expressing gpt were selected by their resistance to growth in mycophenolic acid plus xanthine (3).

Analysis of cytoplasmic RNA from transformed cells.

Cytoplasmic RNA was extracted from semi-confluent cultures of transformed cells (15) and the poly A^+ RNA was isolated with oligo dT cellulose (16). The 5' ends of the gpt RNA were mapped using the Weaver-Weissman variation (17) of the S1 nuclease procedure of Berk and Sharp (18). The DNA hybridization probes used for the S1 mapping were labelled at the 5' ends with ^{32}P-γ-ATP and polynucleotide kinase after cleavage at appropriate restriction sites (19).

Analysis of XGPRT in transformed cells during transient transfections.

Extracts prepared from transformed cells or transiently transfected

cells were assayed for gpt expression by measuring XGPRT production. To normalize the values from independent transient transfections, the cells were simultaneously transfected with a test plasmid containing the E. coli β-galactosidase gene fused to the SV40 early promoter. Extracts, obtained 48 hours after transfection, were analyzed for XGPRT (3) and for β-galactosidase.

RESULTS

Use of the Maize ADH1S Promoter by Monkey Cells

Resistance to G418. Plasmids carrying the neo gene fused to the SV40 early region promoter (pSV2A-neo) or the maize Adh1S promoter (pAdh1S2A-neo) generated clones resistant to G418 (Table 1). The frequency of such transformation was considerably greater than with a plasmid lacking a promoter (pSV02A-neo). This finding suggested that the Adh promoter segment was functioning in the transcription of the neo coding sequence.

As the neo gene fused to the maize Adh1S promoter was expressed sufficiently to yield significant numbers of G418-resistant transformants it was important to determine whether the maize Adh1S promoter was being used in the same manner in the CV-1 cells as in maize. Poly A+ RNA was isolated from transformed lines, hybridized to specifically labelled probe (Fig. 4) and treated with S_1 nuclease in order to map the 5' end of the transcript in CV-1 cells.

The length of fragment transcribed from the maize promoter is predicted to be 27 bp (Fig. 1) if transcripts initiate at the maize start site.

Table 1. Transformation frequencies for recombinant neo plasmids carrying different promoters

Promoter	Plasmid	Transformation frequency	Frequency relative to SV40 promoter
SV40 Early	pSV2A-neo	10^{-4}	100
Adh1S (Maize)	pAdh1S2A-neo	5×10^{-4}	20
0	pSV02A-neo	$<10^{-6}$	<1

Transformation frequency is expressed as the number of cells that produce colonies on selective medium relative to the number of cells plated following transfection with saturating levels of DNA. Results are the averages of 3 experiments.

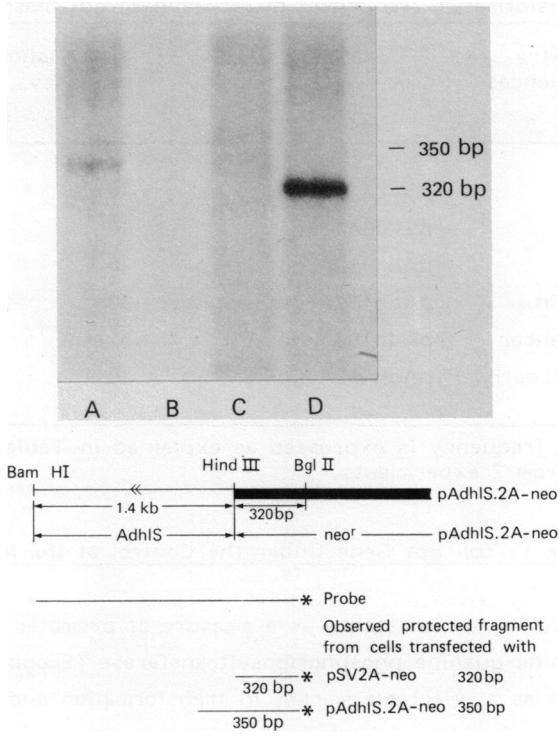

Fig. 4. S1 mapping of the 5' end of RNA extracted from CV1 cells transformed with pSV2A-neo and pAdh1S2A-neo. A DNA probe was prepared by 5' end labelling the BglII restriction site in pAdh1S2A-neo. The probe extended to the BamHI restriction site. Relevant restriction endonuclease cleavage sites are indicated in the diagram. RNA-DNA hybrids were formed under conditions of DNA excess, S1 nuclease digested, and then fractionated on a 4% denaturing acrylamide gel. RNA from cells transformed with A. pAdh1S2A-neo, B. probe alone, C. mock infected, D. pSV2A-neo.

The major protected fragment observed was 350 bp long and included 320 bp of the neo gene and about 30 bp of the maize Adh1S promoter. This length is in agreement with the observed result (Fig. 4). Thus transcripts from pAdh1S-2A-neo gene in CV-1 cells start at the same position as the Adh1S transcript does in maize. The amount of neo-specific poly A+ RNA was about 20 fold greater in the transformants obtained with a plasmid containing an SV40 promoter (pSV2A-neo) than in those obtained with a plasmid containing an Adh promoter (pAdh1S2A-neo) (Fig. 3).

Table 2. Transformation frequences of recombinant gpt plasmids

Promoter	Extra sequences	Plasmid	Transformation frequency	Relative frequency
SV40	-	pSV2A-gpt	10^{-4}	100
0	-	pSV02A-gpt	10^{-7}	-
Adh1S	-	pAdh1S2A-gpt	10^{-6}	1
Adh1S	origin	pAdh1S2A-gpt-ori	10^{-6}	1
Adh1S	enhancer	pAdh1S2A-gpt-EH	5×10^{-4}	18
Adh1S	SV40 early	pAdh1S2A-gpt-SVT	10^{-6}	1

Transformation frequency is expressed as explained in Table 1. Results are averaged from 2 experiments.

Expression of the E. coli gpt Gene Under the Control of the Maize Adh1S Promoter

An alternative to neo resistance as a measure of promoter activity is the E. coli xanthine-guanine phosphoribosyltransferase (Ecogpt) gene which can be used both as a selectable marker in transformation and for assaying gene expression in transient (short term) infection.

Transformation using gpt. CV-1 cells were transfected with the plasmids pSV2A-gpt, pAdh1S2A-gpt and pSV02A-gpt and cells expressing the bacterial gpt gene selected using aminopterin, mycophenolic acid and xanthine. In this case the transformation frequency obtained with the plasmid pAdh1S2A-gpt, (containing the Adh1S promoter) while well above background, was only about 2% that obtained using pSV2A-gpt which contains the SV40 early promoter. This transformation frequency was much lower than the frequency of transformation to neomycin resistance obtained following transfection with pAdh1S2A-neo. A possible explanation for this lower transformation frequency is that the level of expression of the gpt gene required before a cell accumulates enough GPRT to survive the selection regime is higher than that required for the neo gene to survive G418 selection.

A number of other sequences were introduced into the plasmid pAdh1S2A-gpt to monitor effects on transformation frequency (Table 2). The presence of the enhancer sequences of the SV40 promoter (12) increased the transformation frequency by a factor of nearly 20. The

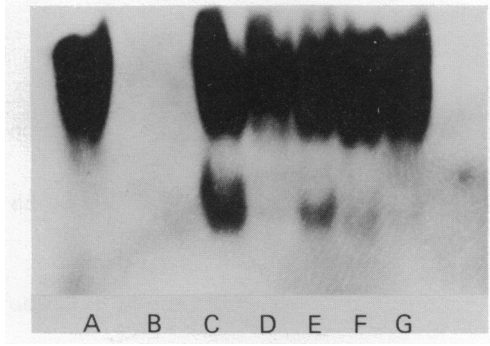

Fig. 5. Detection of XGPRT in protein extracts prepared from COS cells transiently infected with the indicated plasmids. The electrophoresis of cell extracts and in situ assays of XGPRT were as described (1). The plasmids used in the different infections were

A. Mock infected
B. no cells
C. pSV2A-gpt
D. pAdh1S2A-gpt
E. pAdh1S2A-gpt-ori
F. pAdh1S2A-gpt-EH
G. pAdh1S2A-gpt

presence of the SV40 origin (ori) or SV40 origin and early region (SVT) did not increase the transformation frequency.

Expression of the gpt gene in short term (transient) expression studies.

The amount of E. coli XGPRT produced in transfected cells can be assayed enzymatically in protein extracts (1) and the level of XGPRT is an indirect measure of the strength of the promoter transcribing the gpt sequence. Transient expression of gpt can be measured in COS cells where plasmids having an SV40 origin of replication can replicate to high copy number. The results of these studies are shown in Fig. 5 and Table 3.

Table 3. Assay of XGPRT activity in COS cells infected with recombinant gpt

Promoter	plasmids Plasmid	Relative XGPRT activity
SV40 early	pSV2A-gpt	100
0	pSV02A-gpt	0*
Adh1S	pAdh1S2A-gpt	1
+ origin	pAdh1S2A-gpt-ori	20*
+ enhancer	pAdh1S2A-gpt-EH	4
+ SV40 early	pAdh1S2A-gpt-SVT	8

*These plasmids cannot replicate in COS cells
Activity as a percentage of that obtained with the SV40 promoter.

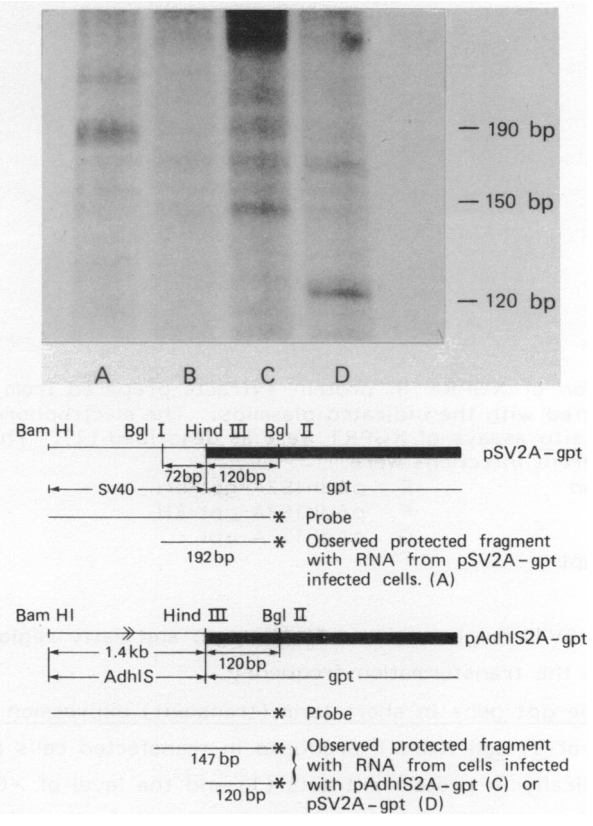

Fig. 6. S1 mapping of 5' end of RNA extracted from transiently infected COS cells. Two infections were set up, in one the cells were infected with pSV2A-gpt and in the other with pAdh1S2A-gpt-SVT. Poly A+ RNA was extracted from each. Two probes were prepared, in both cases cut at the BglII site in the gpt gene and radioactively labelled at the 5' end with ^{32}P-α-ATP and polynucleotide kinase. The probes were the Bam-BglII fragment of pSV2A-gpt and of pAdh1S2A-gpt-SVT. The RNAs were hybridized to the appropriate probe and treated with S1 nuclease.

A. pSV2A-gpt RNA hybridized to pSV2A-gpt probe
B. mock infected
C. pAdh1S2A-gpt-SVT RNA hybridized to pAdh1S2A-gpt-SVT probe
D. pSV2A-gpt RNA hybridized to pAdh1S2A-gpt-SVT probe.

The gpt gene is expressed in constructs with the Adh1S promoter and can be measured in transformation experiments but replication of the plasmid to ensure high copy number is necessary to produce sufficient XGPRT. The effect of copy number on XGPRT levels is greater than that of the enhancer probably because replication produces a large number of copies of the plasmid.

In order to determine that the XGPRT activity resulted from RNA transcripts starting at the same nucleotide in COS cells as in maize, RNA was prepared from cells infected with pAdh1S2A-gpt-SVT and pSV2A-gpt and the 5' termini analysed by S_1 nuclease mapping (Fig. 6). The length of the major protected fragment was as predicted if the maize transcription initiation site is being used. Minor protected fragments are seen indicating that some upstream initiation sites are also being used.

DISCUSSION

Sequence analysis and S_1 mapping of the 5' end of transcripts of plant genes has shown that there are sequence similarities between plant and animal promoters (4,5,7). Bacterial and yeast promoters differ markedly from animal promoters (20, 21). We have shown that the promoter of the maize Adh1S gene can function in monkey cells with transcription being initiated at least some of the time at the same position as in maize. The ability of the maize promoter to function in animal cells raises the possibility that animal promoters may function in plant cells and emphasizes the similarity of plants to other higher eukaryotes. The plant promoter is also modulated by the enhancer sequence from the SV40 promoter. Many promoters show increased expression in the presence of the SV40 enhancer sequences; the level of increase of expression of the Adh1S promoter is 10-20 fold, similar to the increase seen for the β globin promoter. No sequences comparable to the animal cell enhancers have yet been found in plant genomes.

The level of expression of the maize Adh1S promoter could be assayed as up to 20% that of the SV40 early promoter which is itself a strong promoter. The apparent strength of the Adh1S promoter depends upon the method used to assay promoter activity. Frequency of transformation can give a higher measure than level of gene expression.

One possible difference between plant and animal use of the Adh1S promoter is that in plants the transcription of the Adh1 gene is highly regulated. Under aerobic conditions there is little transcription whereas under anaerobic conditions such as flooding, or an argon atmosphere the level of ADH is induced about 50 fold (22). In the animal cell system no anaerobic regulation could be observed. COS cells were transfected with pAdh1S2A-gpt-SVT, divided and grown in a small amount of medium (aerobic conditions) or in a vessel completely full of medium and the lid tightly closed (anaerobic). These latter conditions are sufficiently

anaerobic to induce ADH1 fully in maize tissue culture. No increase in XGPRT activity was seen under anaerobic conditions showing that either the gene is fully induced under the normal "aerobic conditions" or else it is not regulated in the animal cell system. In maize, a set of proteins called transition proteins is made within 15 minutes of anaerobic treatment (22) and ADH activity only becomes apparent more than one hour after induction. If the transition proteins are necessary for the induction of the Adh1 mRNA in maize, their absence from animal cells would result in absence of the anaerobic response. Alternatively the conditions of growth in tissue culture are sufficiently anaerobic to keep the promoter on continuously and we were not able to detect the uninduced state. The determination of the sequences responsible for the anaerobic induction of alcohol dehydrogenase awaits a homologous system permitting a functional analysis of the promoter by deletions and directed mutagenesis.

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