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Regulated expression of an alcohol dehydrogenase 1 chimeric gene introduced into maize protoplasts

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Abstract. Transient-expression analysis has shown anaerobic regulation of the alcohol dehydrogenase (*Adh1*) promoter in a chimeric construct. Chimeric plasmids containing the promoter for the *Adh1* gene of maize (*Zea mays* L.) linked to the coding sequence of the chloramphenicol acetyl transferase (*cat*) gene were introduced into maize protoplasts using electroporation. Both the introduced *Adh1* promoter and the endogenous *Adh1* gene promoter are regulated at the RNA level, the O_2-tension optimum for induction is the same for both, and both promoters initiate transcription from the same site. The demonstration of regulation of a plant gene promoter in a transient expression system will allow the identification of *cis* acting sequences responsible for regulation.

Key words: Alcohol dehydrogenase – Anaerobiosis – Electroporation – Gene regulation – Protoplast – Transient expression – *Zea* (gene expression).

Introduction

Transformation of plant genes into a number of dicotyledonous plants (e.g. tobacco, petunia) has permitted the recognition of both constitutive and inducible plant gene promoters (Timko et al. 1985; Herrera-Estrella et al. 1983). Until recently, cereal gene promoters could be studied only in dicotyledonous systems, because of the lack of an efficient method for transferring genes into a monocotyledonous plant (e.g. wheat, maize). The recent introduction of electroporation as a technique for direct DNA transfer into monocotyledonous protoplasts, either transiently or stably (Potrykus et al. 1985; Fromm et al. 1985), now permits the analysis of monocotyledonous genes in a homologous system. In this technique the application of a high-voltage electric pulse to protoplasts facilitates the uptake of DNA. We report the expression and regulation of an inducible monocotyledonous gene promoter in a homologous transient-expression system, following its introduction using electroporation.

The maize alcohol dehydrogenase 1 (ADH) enzyme is one of about 20 polypeptides which are induced in response to anaerobic stress (Sachs et al. 1980). At the onset of anaerobiosis, e.g. in flooded roots, aerobic protein synthesis is rapidly repressed and within 5 h, anaerobic polypeptides constitute about 70% of newly synthesized protein (Sachs et al. 1980). Previous studies on expression of the *Adh1* gene of maize have shown that regulation is at the transcriptional level, as evidenced by nuclear run-off (Vayda and Freeling 1986) and by steady-state mRNA levels (Gerlach et al. 1982). Anaerobic regulation also has a translational component; synthesis of aerobic proteins is repressed during anaerobiosis, although the mRNAs encoding them remain translatable in an in-vitro system for at least 24 h after initiation of anaerobiosis (Gerlach et al. 1982). In this paper we demonstrate that the 1,200 base pairs upstream of the *Adh1* coding region contain sequences sufficient for anaerobically regulated expression.

Materials and methods

Construction of plasmid DNAs. pCaMVCAT was obtained from M. Fromm (Stanford University Stanford, Cal., USA; see Fromm et al. 1985) and contains the 35S promoter of cauliflower mosaic virus (Franck et al. 1980) linked to the chloramphenicol acetyl transferase (*cat*) gene coding sequence from Tn9 and the 3′ sequences of nopaline synthase (*Nos*) (Bevan et al. 1983). pNosCAT411 is a derivative of pNCAT4 (obtained from L.

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Abbreviations: ADH = alcohol dehydrogenase; *adh* = ADH genes; CAT = chloramphenicol acetyl transferase enzyme; *cat* = CAT gene; *Nos* = nopaline synthase gene

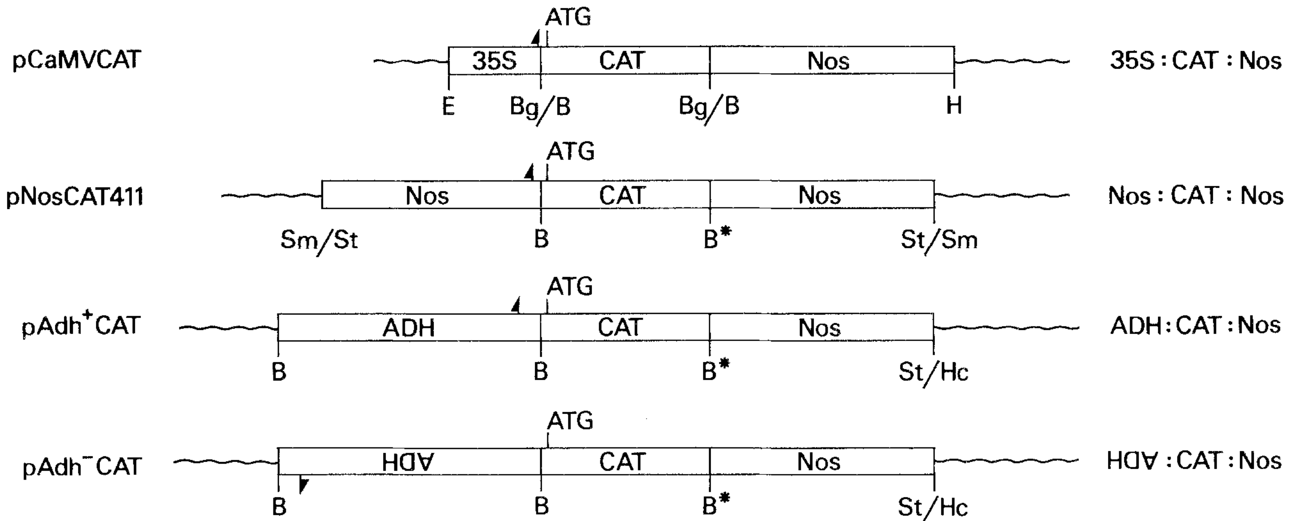

Fig. 1. Plasmid DNAs used for transient-expression studies in maize protoplasts. The structure of the chimeric genes contains different promoters linked to the *cat* reporter gene and 3′ regulatory regions of the *Nos* gene. The *flags* in the line drawings indicate the transcription start. The *asterisk* indicates the loss of a restriction site. *E* indicates an EcoRI site; *B* a BamHI site; *Bg* a BglII site; *H* a HindIII site; *Sm* a SmaI site; *St* a StuI site; and *Hc* a HincII site

Herrera-Estrella and P. Zambryski, Rijksuniversiteit, Gent, Belgium) in which the 3′ BamHI site linking the CAT coding region to the 3′ *Nos* sequences has been eliminated by filling in with the large fragment of DNA polymerase I and religating. This 3′ BamHI$^-$ pNCAT4 plasmid was digested with StuI, which cuts at nucleotide 89 and at approximately nucleotide 3000 (see Bevan et al. 1983), and blunt-end ligated into the SmaI site of pUC18 to yield pNosCAT411. Another derivative of 3′ BamHI$^-$ pNCAT4 (pCN100), containing only the *cat* and 3′ *Nos* sequences, was constructed by cloning the BamHI-StuI fragment of 3′ BamHI$^-$ pNCAT4 into the BamHI-HincII site of pUC18. The *Adh1* promoter was produced by Bal31 deletion from the HindIII site in the first intron of the maize *Adh1S* gene (Dennis et al. 1984). BamHI linkers were ligated onto the deletion end points, following treatment of the DNA with the large fragment of DNA polymerase I, and then cloned into pUC18 as a BamHI fragment from the BamHI site (−1,096 with respect to transcription start) of *Adh1S* to the BamHI linker at the deletion endpoint. The promoter deletions were characterized by restriction-endonuclease mapping and DNA-sequence analysis. A selected fragment containing bases −1,096 to +106 of the maize *Adh1S* gene flanked by BamHI linkers was cloned into the BamHI site in front of the *cat* DNA sequences in pCN100. This cloning step yielded two types of plasmids, pAdh$^+$CAT in which the *Adh1* promoter is in the correct orientation with respect to *cat*, and pAdh$^-$CAT in which the *Adh1* promoter is in the reverse orientation (Fig. 1).

Cell lines and culturing conditions. The maize (*Zea mays* L.) cell suspension line Black Mexican Sweet XII-II (BMS) (Chourey and Zurawski 1981) was grown in Murashige-Skoog-Ed Green (MSEG) medium (Green and Phillips 1975) at 26° C, shaking at 100 rpm. Protoplasts were isolated as described by Potrykus et al. (1979).

Electroporation and CAT assays. For electroporation experiments, 1 ml of protoplasts were placed in 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes), pH 7.2, 150 mM NaCl, 0.2 M mannitol at $3 \cdot 10^6$ protoplasts/ml. Plasmid DNA used for the transfection experiments was twice-purified over CsCl equilibrium gradients to eliminate any possibility of RNA contamination. The DNA of interest was added to the protoplasts prior to a heat-shock treatment at 45° C for 5 min. The protoplasts were then placed on ice for 5 min and electroporated by a single 50-ms pulse at 250 V, using a capacitor-discharge electroporation apparatus (for description, see Fromm et al. 1985), on ice. The electroporated protoplasts were left on ice a further 10 min, then diluted to $3 \cdot 10^5$ protoplasts/ml with protoplast culture medium (PCM; Chourey and Zurawski 1981). After incubation at 26° C for 20 h in the dark, sonicated extracts of the transfected protoplasts were made and protein levels measured as described in Howard et al. (1985). Samples used for CAT assays were normalized with respect to protein content to eliminate variability due to differential recovery of protoplasts. The CAT assays were done as described by Gorman (1982) and the acetylated products of the reaction resolved from the non-acetylated chloramphenicol by thin-layer chromatography (TLC) on a silica plate in methanol:chloroform (95:5, v/v). The thin-layer plate was enhanced by fluorography in 2-methyl naphthalene containing 0.4% 2,5-diphenyloxazole (PPO), dried, and exposed to X-ray film (Fujifilm RX, Fuji Photo Film Co., Tokyo, Japan) at −80° C for 1–4 d. To quantitate the assays, the radioactive acetylated derivatives were cut from the TLC plates and counted by liquid scintillation. The values given represent the mean of at least three independent experiments. Results varied by less than twofold between experiments.

Adjustment of oxygen tension. Immediately after isolation, protoplasts were placed in mason jars (800 ml) which were subsequently sealed and adjusted to 1%, 5% and 10% O_2, as described by Hanson and Jacobsen (1984). The jars were routinely assayed by gas chromatography to verify estimated O_2 levels. Protoplasts used for the 20% O_2 sample were incubated at atmospheric oxygen level. Electroporated protoplasts were incubated in the different O_2 environments for 20 h and then extracted.

Isolation of RNA and S1 mapping. RNA was isolated by the method of Taylor and Powell (1982). Prior to use for S1 map-

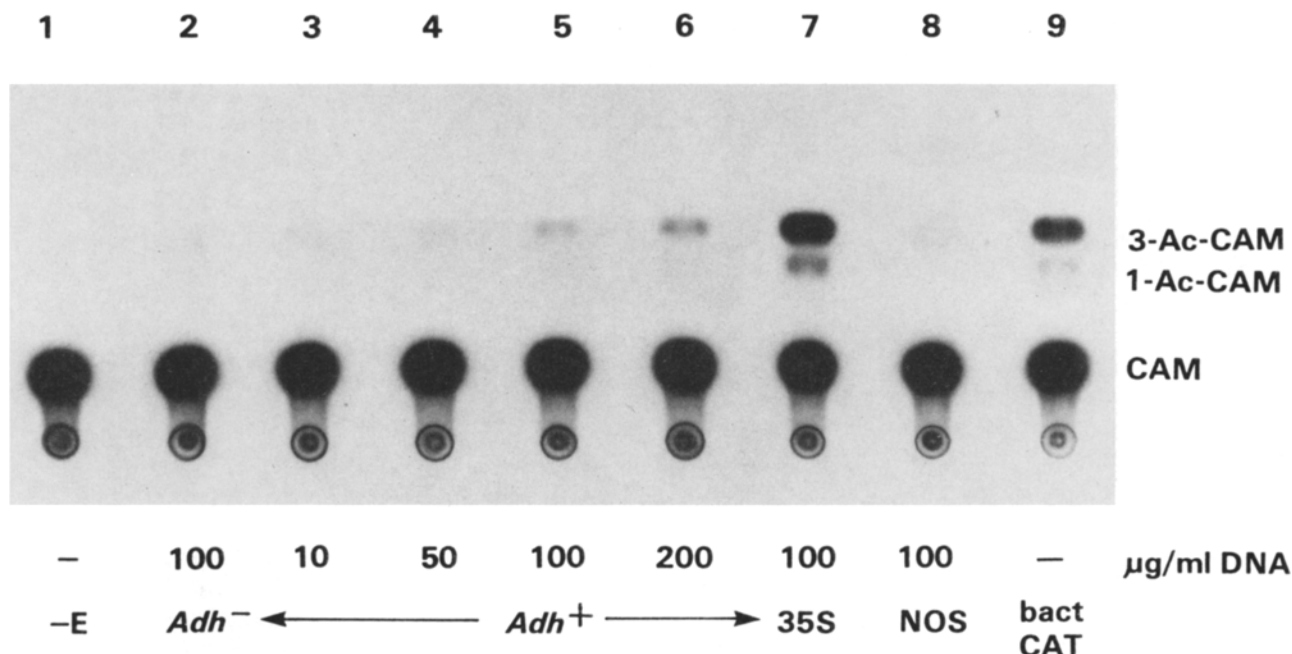

Fig. 2. Relative promoter activity of different *cat* constructs. The CAT assays were of non-electroporated protoplasts (*lane 1*), protoplasts transfected with 100 µg/ml pAdh$^-$CAT (*lane 2*), protoplasts transfected with either 10 µg/ml (*lane 3*), 50 µg/ml (*lane 4*), 100 µg/ml (*lane 5*) or 200 µg/ml (*lane 6*) of pAdh$^+$CAT, and protoplasts transfected with 100 µg/ml pCaMVCAT (*lane 7*) or 100 µg/ml pNosCAT411 (*lane 8*). In *lane 9*, $2.5 \cdot 10^{-3}$ units of bacterial CAT enzyme alone was added to the enzyme assay. Positions of the chloramphenicol and its acetylated derivatives are indicated on the side of the figure

ping, the RNA was routinely digested with two units RNAase-free DNAaseI (Promega Biochemicals, Madison, Wis., USA) per 50 µg RNA. A 25-base-pair (bp) oligonucleotide primer, complementary to the coding strand of *cat*, was annealed to the 482 bp *PstI*/*EcoR1* fragment of pAdh$^+$CAT (see bottom of Fig. 4), labelled by primer extension (Hudson et al. 1982), digested with *PstI*, and the labelled strand purified on a sequencing gel. This strand was then hybridized to 50 µg of total RNA isolated from the protoplasts transfected with either pAdh$^+$CAT or pAdh$^-$CAT treated with S1 nuclease, as described by Weaver and Weissman (1979), and run on a sequencing gel.

Results and discussion

Promoter activities in maize at atmospheric oxygen level. To establish conditions for analyzing transient expression, chimeric genes (Fig. 1) with promoters derived from the 35S gene of cauliflower mosaic virus (pCaMVCAT) (Fromm et al. 1985), the nopaline synthase (*Nos*) gene of *Agrobacterium tumefaciens* (pNosCAT411) (Bevan et al. 1983), or the *Adh1S* gene of maize (pAdh$^+$CAT) were assayed under atmospheric oxygen levels (20%). The promoters were joined to the coding region of the chloramphenicol acetyl transferase gene (*cat*). The 3′ processing signals were provided by the 3′ region of the *Nos* gene. As a control the reverse orientation of the *Adh1* promoter linked to the same *cat*/*Nos* 3′ cassette (pAdh$^-$CAT) was also made.

When pCaMVCAT was introduced by electroporation into maize protoplasts (lane 7, Fig. 2), the level of CAT expression was approx. 20-fold above the level of CAT activity detected in maize protoplasts which were not electroporated (lane 1, Fig. 2). Expression of this construct in maize has been reported previously, as has expression controlled by the *Nos* promoter (Fromm et al. 1985). We were not able to detect any expression significantly greater than background when pNosCAT411 was introduced in several independent experiments (lane 8, Fig. 2). When pNosCAT411 was transiently introduced into tobacco (J. Ellis, CSIRO Division of Plant Industry, personal communication), a strong CAT signal was detected, indicating that the construct is capable of expression. The non-expression of pNosCAT411 in maize in our experiments may be the consequence of several reasons, such as the cell line used or the level of anaerobiosis; it is not necessarily a contradiction of the previous report (Fromm et al. 1985). When the pAdh$^+$CAT was introduced into maize protoplasts, the level of CAT activity following transfection was correlated with the concentration of input pAdh$^+$CAT DNA (lanes 3–6, Fig. 2). Using 200 µg of pAdh$^+$CAT DNA/ml of protoplasts, the highest level tested, there was an approx. fourfold higher level of CAT expression than back-

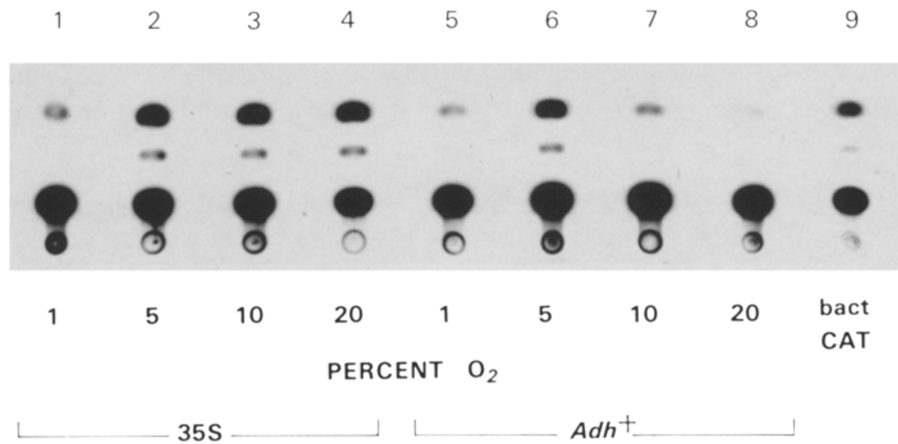

Fig. 3. Effect of O_2 tension on promoter activity of different *cat* constructs. Electroporated protoplasts were incubated in the different O_2 environments for 20 h and extracted and assayed for CAT activity. Protein-normalized samples for CAT assays were taken from protoplasts transfected with 100 μg/ml pCaMVCAT (*lanes 1–4*) and placed in 1% (*lane 1*), 5% (*lane 2*), 10% (*lane 3*) or 20% (*lane 4*) O_2; and from protoplasts transfected with 100 μg/ml pAdh$^+$CAT (*lanes 5–8*) and placed in 1% (*lane 5*), 5% (*lane 6*), 10% (*lane 7*) or 20% (*lane 8*) O_2

ground. At 100 μg of pAdh$^+$CAT/ml of protoplasts there was an approx. twofold higher level of CAT expression than background. Experiments using pAdh$^-$CAT, the reverse-orientation promoter region, as the input DNA (lane 2, Fig. 2) resulted in only background levels of CAT activity. The CAT activity was therefore dependent on the concentration of the input DNA and the orientation of the *Adh1* promoter.

Anaerobic induction of endogenous Adh1 in tissue culture. Expression of the *adh* gene is normally induced during anaerobiosis (Hageman and Flesher 1960). The protoplasts used for these experiments are derived from suspension-cell cultures and may be regarded as being in a continuous state of flooding (i.e. at least partially anaerobic). This probably accounts for the endogenous *Adh1* gene being partially expressed in the absence of additional anaerobic induction. We sought to establish conditions whereby we could observe regulation of the level of *Adh1* gene expression, and assayed expression of the endogenous *Adh1* gene under the normal culture conditions and under reduced O_2 tensions. RNA was extracted from protoplasts incubated at 1%, 5%, 10% or 20% (i.e. atmosphere) O_2. Oxygen concentrations of 5% and 10% resulted in increased amounts of *Adh1*-specific message relative to levels present under atmospheric conditions. Levels of expression decreased at 1% O_2, which we believe, from cytological observations, to be the result of cell death and general loss of metabolic vigour (data not shown; for evidence of anaerobic regulation of the endogenous *Adh1* gene see Fig. 4).

Anaerobic induction of the introduced Adh1 promoter. Having established conditions for maximizing the anaerobic induction of the endogenous maize *Adh1* gene in tissue culture, we tested whether the introduced *Adh1* promoter linked to the *cat* gene showed a similar response. Oxygen concentrations of 5% or 10% resulted in increased levels of CAT activity in maize protoplasts transfected with pAdh$^+$CAT (Fig. 3, lanes 5–8). At 5% O_2 (lane 6), using 100 μg of pAdh$^+$CAT/ml of protoplasts, CAT activity is approx. fourfold higher than under atmospheric O_2 levels. At 1% O_2 (lane 5), CAT activity drops to about the level detected under atmospheric conditions. The introduced *Adh1* chimeric construct is expressed optimally at the same O_2 levels as the endogenous *Adh1* gene.

To ensure that the increased CAT activity was the result of anaerobic induction specific to the *Adh1* promoter, protoplasts transfected with pCaMVCAT were incubated in the different O_2 environments. Levels of CAT activity did not vary with O_2 tension, except at the 1% O_2 level where there was decreased expression (Fig. 3, lanes 1–4). This result supports the conclusion that the anaerobic induction of CAT enzyme activity is a property of the *Adh1* promoter.

Regulation of the Adh1 promoter reflects endogenous Adh1 induction at the RNA level. To establish whether the normal transcription-initiation site was used during the transient expression of pAdh$^+$CAT, RNA was isolated from transfected protoplasts and analyzed by S1 mapping. The results demonstrate that transcription initiates in the pAdh$^+$CAT promoter region at the same site as it does in the endogenous maize gene (Fig. 4, panel A). Protoplasts transfected with pAdh$^+$CAT and expressed at either 5% or 20% O_2 were also analyzed by S1 mapping. The data showed that the induced regulation of pAdh$^+$CAT is at the

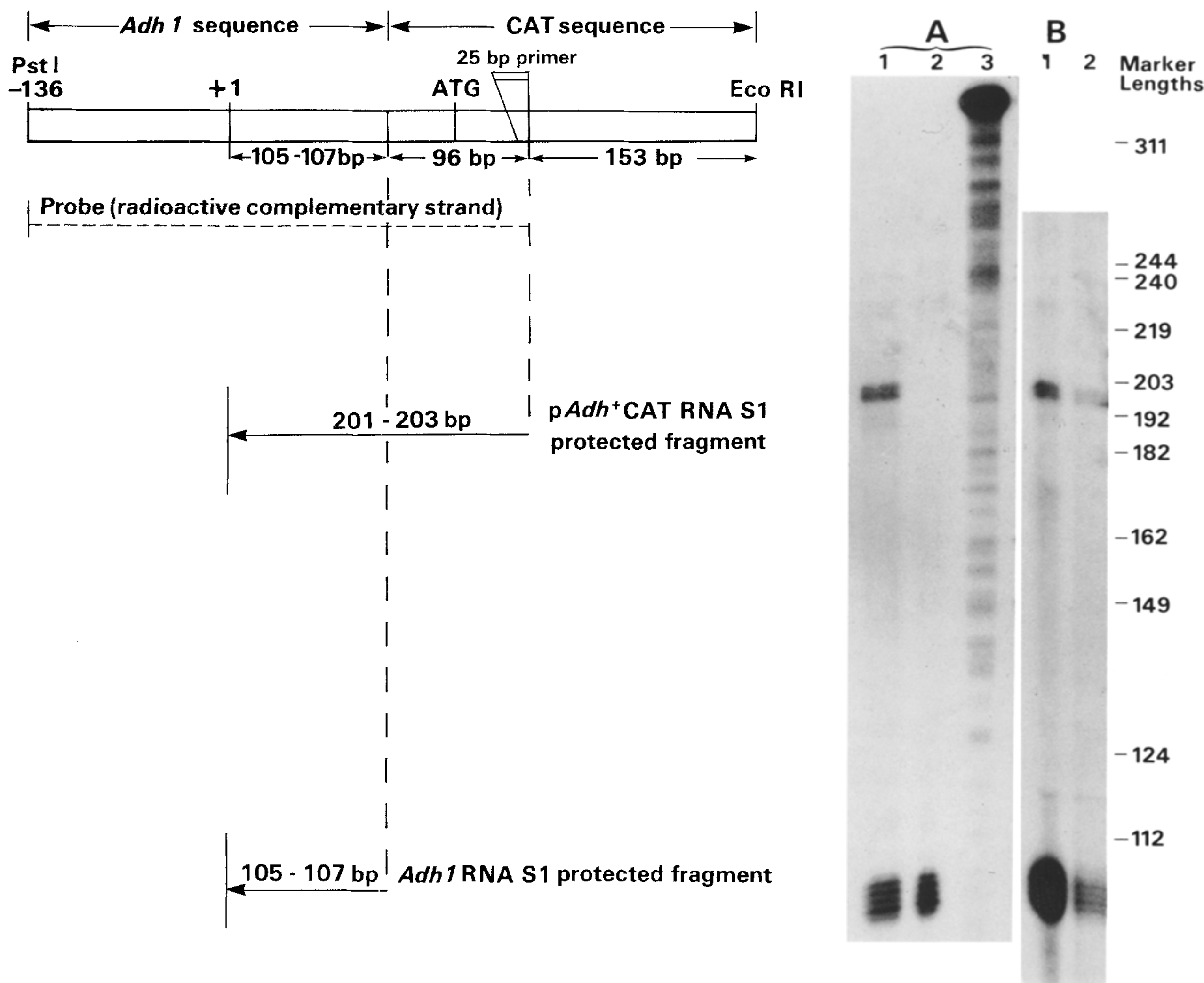

Fig. 4. S1 nuclease mapping of the transcription start of pAdh$^+$CAT. S1 nuclease mapping was done on 50 μg RNA isolated from $3 \cdot 10^7$ protoplasts which had been transfected with 200 μg/ml of pAdh$^+$CAT (*lane 1, panel A*) or 200 μg/ml of pAdh$^-$CAT (*lane 2, panel A*) and incubated for 20 h at 5% O_2. The probe used for the S1 mapping is shown at the top of the figure and is described in *Materials and methods*. *Lane 3* (*panel A*) was loaded with untreated probe. In a separate experiment, 50 μg of RNA isolated from protoplasts transfected with 200 μg/ml pAdh$^+$CAT which had been incubated at either 5% (*lane 1, panel B*) or 20% (*lane 2, panel B*) O_2, were analyzed by S1 nuclease mapping. Marker sizes of end-labelled MspI cut pBR322 are indicated on the side of the figure. As the probe contains both *Adh1* leader sequence and CAT coding sequence, both endogenous *Adh1* mRNA and mRNA from the chimeric construct protect fragments. The two protected DNA fragments are distinct as only the *Adh1* leader sequences of the labelled fragment are protected by the endogenous *Adh1* mRNA, whereas *Adh1* and *cat* sequences are protected by the mRNA from the chimeric construct

transcription level, and parallels that for the endogenous *Adh1* gene (see Fig. 4, panel B).

Concluding remarks. Our experiments demonstrate the expression and normal regulation of an inducible promoter from a monocotyledonous gene when electroporated into protoplasts derived from a homologous cell culture system. The *Adh1* gene of maize is anaerobically regulated. Our experiments show that the *Adh1* promoter region, when introduced into maize protoplasts without the coding and 3′ regions of the *Adh1* gene, is sufficient for anaerobic induction of the gene. Furthermore, the anaerobic regulation of the introduced *Adh1* promoter parallels the anaerobic regulation of the endogenous *Adh1* gene, both with respect to O_2-tension optimum and inducibility at the RNA level. Through the use of the transient-expression system described here, we are now in the process of identifying the particular sequences in the *Adh1*

upstream region responsible for anaerobic response.

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