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Establishment of a System for Conditional Gene Expression Using an Inducible tRNA Suppressor Gene

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We investigated the use of the prokaryotic tetracycline operator-repressor system as a regulatory device to control the expression of *Dictyostelium discoideum* tRNA genes. The *tetO*$_1$ operator fragment was inserted at three different positions in front of a tRNA^{Glu}(Am) suppressor gene from *D. discoideum*, and the tetracycline repressor gene was expressed under the control of a constitutive actin 6 promoter. The effectiveness of this approach was determined by monitoring the expression of a β-galactosidase gene engineered to contain a stop codon that could be suppressed by the tRNA. When these constructs were introduced into *Dictyostelium* cells, the repressor bound to the operator in front of the tRNA gene and prevented expression of the suppressor tRNA. Addition of tetracycline (30 μg/ml) to the growth medium prevented repressor binding, allowed expression of the suppressor tRNA, and resulted in β-galactosidase synthesis. The operator-repressor complex interfered with tRNA gene transcription when the operator was inserted immediately upstream (position +1 or −7) of the mature tRNA coding region. Expression of a tRNA gene carrying the operator at position −46 did not respond to repressor binding. This system could be used to control the synthesis of any protein, provided the gene contained a translational stop signal.

tRNA suppressors are a classic means of regulating the expression of a protein. In the absence of a suppressor tRNA, the mRNA derived from a gene which has been mutated to contain a stop codon will be incompletely translated. When a tRNA suppressor gene is introduced into the cell, an authentic protein is synthesized, provided the suppressor tRNA inserts the same amino acid as the cognate tRNA of the unmutated codon. This is a powerful tool for analysis of the function of any gene but has been relegated primarily to procaryotes because of the difficulty of manipulating tRNA expression in eukaryotic cells. Eukaryotic tRNA genes contain gene-internal polymerase III (polIII) promoters (for reviews, see references 23 and 46). These transcriptional control regions become part of the mature tRNA coding sequence and are indispensable for tRNA function. As a consequence, these regions are difficult to manipulate. Another consequence is that other than in the case of polII genes, polIII gene promoters cannot be replaced by regulated polII promoters, such as the heat shock and metallothionein promoters.

In addition to the gene-internal control elements, 5′-flanking regions of tRNA genes frequently exert a modulatory influence on gene expression (1–3, 10, 15, 17, 27, 37, 41, 47, 49). Although the mechanism of tRNA gene modulation by 5′-flanking regions is not understood, we recently demonstrated that stable binding of a protein near a tRNA gene strongly inhibits its expression (39). This inhibition apparently resulted from masking of the initiation site for tRNA gene transcription by the bound protein and from a strong interaction of the protein with the RNA polIII transcription complex. These findings suggested that control of tRNA transcription might be possible with the use of a DNA-binding protein whose affinity to a particular DNA target sequence can be controlled.

Very well-studied DNA-binding proteins include the prokaryotic repressor proteins which are involved in regulating certain operons, like the *lac* operon or the *tet* operon (42). These proteins recognize and bind with very high affinity to specific target sequences, known as operators. It has been shown that prokaryotic repressor-operator systems can also negatively regulate transcription from eukaryotic polII promoters (8, 21, 22, 29).

In this study, we evaluated the ability of a prokaryotic repressor-operator system to control the transcription of a *D. discoideum* tRNA gene in vivo. We used genetic elements which control the expression of tetracycline resistance in bacteria. The Tn*10*-encoded Tet repressor regulates the expression of its own gene and that of the tetracycline resistance gene by binding to two operators which overlap with two divergent promoters (5, 26). Only in the presence of tetracycline can these promoters be recognized by RNA polymerases, since tetracycline acts as a specific inducer, preventing the repressor from binding to its operator sequence.

To adapt this control system for regulation of eukaryotic tRNA gene transcription, the following manipulations were required. (i) A *tet* operator fragment had to be inserted in front of a tRNA gene, preferably at a region which overlaps with the transcription initiation site. (ii) The repressor gene had to be engineered in such a way that it could be expressed in a eukaryote. (iii) The tRNA gene had to be rendered a suppressor tRNA gene, allowing monitoring of its expression.

These components were assembled by using the eukaryotic amoeba *D. discoideum*. This organism provides a well-established genetic system (38), including suppressor genetics (18). As a reporter for nonsense suppression, a mutated version of the *lacZ* gene from *Escherichia coli* which can be

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expressed in *D. discoideum* as controlled by a homologous actin 6 promoter is available (18, 19). Amber suppression is very efficient in *D. discoideum*, and suppressor tRNA genes seem not to interfere significantly with cell growth. This is a special advantage of *D. discoideum*, presumably conferred by the infrequent use of amber stop codons in this organism. The extreme A-T-rich codon bias leads to nearly exclusive use of the ochre codon UAA as a translational stop signal (45).

The general usefulness of an inducible amber suppressor tRNA gene is demonstrated by conditionally shifting cells from a β-gal$^-$ to a β-gal$^+$ phenotype and back again. It is reasonable to assume that this system can now be applied to the expression of any eukaryotic gene that can be engineered to contain an amber stop codon. Not only can this system be used to express new proteins in *D. discoideum*, but combined with homologous gene replacement, endogenous genes can be replaced with ones in which expression can be regulated.

MATERIALS AND METHODS

Plasmid DNAs. Plasmid pDneoA6PTR was obtained after in-frame fusion of the tetracycline repressor gene to the N-terminal coding region of the actin 6 gene contained on pDneo2 (56). The resulting plasmid contains, in addition to the actin 6::*tetR* fusion, a neomycin resistance gene whose expression in *D. discoideum* is controlled by the homologous actin 15 control regions (see Fig. 1).

The tRNA^{Glu}(Am) suppressor used in this study is a derivative of *glu2*(UUC), which resides on a 1.15-kb genomic *Eco*RI fragment from *D. discoideum* (13). The 5′-UUC-3′ anticodon sequence was changed to 5′-CUA-3′ by primer-directed in vitro mutagenesis (18). Variants of the resulting amber suppressor with deletions of the 5′-flanking region up to nucleotides −46, −7, and +1 were obtained with the processive exonuclease BAL 31. These genes served as recipients of the oligonucleotide 5′-GACTCTAGAC TCTATCAATGATAGAGTcTAGAGTC-3′, whose underlined 19-bp core sequence corresponds to the *tetO1* operator of transposon Tn*10*. The resulting plasmids were termed pGTET+1, pGTET−7, and pGTET−46 (see Fig. 3).

Construction of plasmid pA6PTlac.1 has already been described (19). This plasmid contains a fusion of the actin 6 gene from *D. discoideum* and the *lacZ* gene from *E. coli*. Transcription termination is controlled by the actin 8 terminator from *D. discoideum*. A GAG glutamic acid codon in the N-terminal region of the actin 6::*lacZ* fusion gene was changed into a UAG amber codon by primer-directed mutagenesis (18). A *Hin*dIII fragment containing the actin 6::*lacZ*(Am) fusion was isolated and ligated into the three pGTET plasmids, yielding pGTETR+1, pGTETR−7, and pGTETR−46 (see Fig. 3).

Transformation. *D. discoideum* Ax-2 cells were routinely grown in HL5 medium (53) at 22°C. Transformants were obtained by cotransformation with pDneoA6PTR (6 μg) and with one of the pGTETR plasmids (6 μg) and selected in the presence of 15 μg of G418 per ml (20, 40). Primary colonies were picked from the petri dish and recloned on SM agar plates with *Klebsiella aerogenes* (54). After 2 days of growth at 22°C, single colonies were transferred to axenic medium and subsequently tested for β-galactosidase activity.

Western blotting (immunoblotting)–β-galactosidase assay. Expression of the Tet repressor protein was determined by Western blot analysis. A 50-μg sample of whole-cell protein was size fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a GeneScreen Plus membrane. Monoclonal antibody mctet6 (kindly provided by Thomas Winckler) was used to detect the recombinant Tet repressor protein. The amount of repressor protein per cell was estimated by comparing the intensity of staining of the whole-cell extracts with known amounts of authentic Tet repressor protein.

β-Galactosidase was measured essentially as previously described (19). Cells were harvested by centrifugation (500 × *g*, 5 min) and washed twice in phosphate buffer (14.7 mM KH_2PO_4, 2 mM Na_2HPO_4, pH 6). After a freeze-thaw cycle with 5×10^6 to 5×10^7 cells in 1 ml of phosphate buffer, the suspension was vortexed for 1 min and cleared by centrifugation for 5 min in an Eppendorf centrifuge. A 2-μl volume of extract was used to determine protein concentrations by the dye-binding method (Bio-Rad). Enzyme activity was assayed from 100 μl of extract. A 300-μl volume of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7), 50 mM β-mercaptoethanol, and 200 μl of a solution containing 4 mg of *o*-nitrophenyl-β-D-galactopyranoside per ml in 100 mM phosphate buffer (pH 7) were added, and assays were incubated at 22°C. Reactions were stopped by adding 400 μl of 1 M Na_2CO_3 and cleared for 5 min in a microcentrifuge. A 500-μl aliquot was diluted with 500 μl of Z buffer, and the optical density was determined at 420 nm. Specific enzyme activities are given as katals per milligram of protein. One katal is defined as the enzymatic activity which hydrolyzes 1 mol of *o*-nitrophenyl-β-D-galactopyranoside per s at 22°C.

Primer extension analysis. Isolation of a low-molecular-weight RNA fraction containing tRNAs has already been described (12). Ten micrograms of RNA was coprecipitated with 10 pmol of the 5′-labeled primer fragment DD8 (5′-CCAGTGTTAGAGAC-3′), which anneals to nucleotides 30 to 43 of tRNA^{Glu}(Am). Dry pellets were dissolved in 10 ml of buffer containing 50 mM KCl and 50 mM Tris-HCl (pH 8), and incubated for 5 min at 90°C and for 10 min each at 0°C and room temperature. Five microliters of a buffer containing 50 mM Tris-HCl (pH 8), 50 mM KCl, 20 mM $MgCl_2$, 6 mM dithiothreitol, 2.5 mM deoxynucleoside triphosphates, and 3 U of avian myeloblastosis virus reverse transcriptase was added. Assays were incubated at 50°C for 1 h and terminated by ethanol precipitation. Under these conditions, the DD8 primer exclusively recognizes mutant tRNAs, even in the presence of a large excess of wild-type tRNA^{Glu}(TTC). The pellets were dissolved in 1.5 μl of sterile distilled water and 2 μl of loading buffer (95% formamide, 10 mM EDTA, 0.1% bromphenol blue, 0.1% xylene cyanol). Samples were boiled for 5 min and chilled on ice, and products were separated on 8% polyacrylamide gels containing 50% urea (44).

RESULTS

The bacterial tetracycline repressor can be expressed in *D. discoideum*. The tetracycline repressor is a DNA-binding protein with high affinity for the tetracycline operator. To allow expression of *tetR* in *D. discoideum*, the gene was fused in frame to the actin 6 promoter from *D. discoideum*. The resulting plasmid, pDneoA6PTR, is 7kb long and contains, in addition to the *tetR* gene, a neomycin resistance gene under *Dictyostelium* promoter control, allowing positive selection for transformants (Fig. 1).

Transgenic *D. discoideum* strains with stably integrated and amplified pDneoA6PTR plasmids efficiently expressed the Tet repressor (Fig. 2), regardless of whether the cells

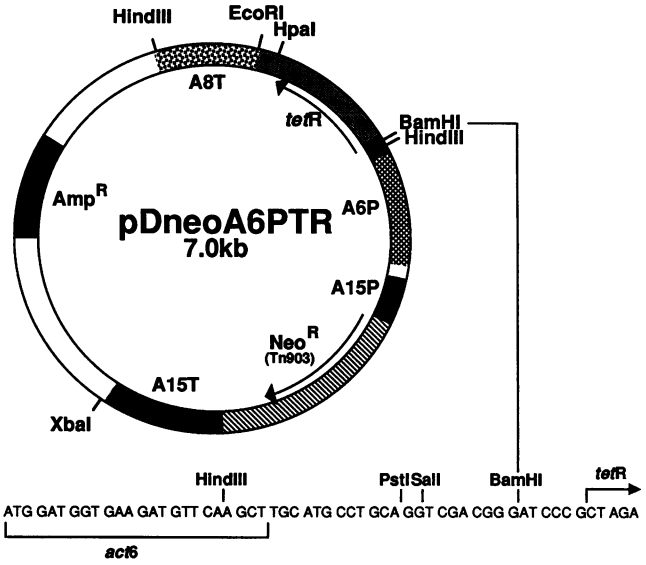

FIG. 1. Plasmid pDneoA6PTR is a derivative of pDneo2 (56). It contains the neomycin resistance gene (Neo^R) derived from Tn*903* under actin 15 transcriptional control (A15P, A15T) and a fusion of the N-terminal region of the actin 6 gene (A6P) with the *tetR* gene from Tn*10*. Transcription of this gene terminates in the actin 8 terminator (A8T). Owing to the cloning strategy, the first three amino acids of the bacterial repressor were removed. Furthermore, the fusion gene is slightly larger than the bacterial *tetR* gene.

were grown with or without tetracycline. The fusion protein was slightly larger (25 kDa) than the authentic *E. coli* repressor (23 kDa), owing to the 17 actin codons to which the gene was fused. Nevertheless, this slightly altered repressor protein retained its characteristic activity. In gel retardation assays, a specific protein-DNA complex was detected when nuclear extracts prepared from transgenic *D. discoideum* strains expressing the bacterial Tet repressor were incubated with a labelled *tet* operator fragment (data not shown). Depending on the copy number in individual transformants, the number of Tet repressor proteins was estimated to about 100,000 to 250,000 molecules per cell (data not shown).

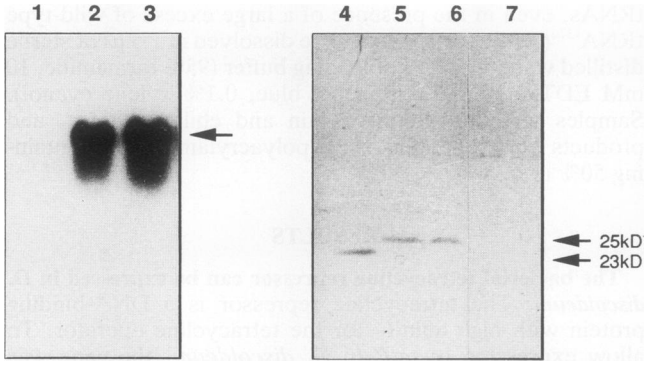

FIG. 2. Expression of the Tet repressor in *D. discoideum*. RNAs were prepared from Ax-2 cells (lane 1) and pDneoA6PTR transformants grown without (lane 2) and with (lane 3) tetracycline. These RNAs were probed with a *tetR*-specific oligonucleotide. Assays 4 to 7 represent Western blot analysis of protein isolated from tetracycline-resistant *E. coli* cells (lane 4), from *D. discoideum* cells transformed with pDneoA6PTR (lanes 5 to 6), or from untransformed *D. discoideum* cells (lane 7). Transformants were grown either without (lane 5) or with (lane 6) tetracycline.

Suppressor tRNA gene expression can be controlled by the *tetR* gene product but depends on the presence of a *tet* operator and its position relative to the mature tRNA coding region. A $tRNA^{Glu}$(CUA) suppressor gene from *D. discoideum* has been shown to serve as an efficient template that produces active suppressor tRNAs (18). Three derivatives of this gene, obtained after BAL 31 deletion of the 5′-flanking nucleotides, were chosen for insertion of a synthetic oligonucleotide which contains as its central core the 19-bp palindromic *tetO1* operator from transposon Tn*10*. The oligonucleotide whose sequence is depicted in Fig. 3 was inserted at positions +1, −7, and −46 relative to the mature tRNA coding region. The reporter gene used to monitor suppression was a bacterial *lacZ* gene that had been altered to contain an amber stop codon in its N-terminal coding part (18). The resulting plasmids, pGTETR+1, pGTETR−7, and pGTETR−46 (Fig. 3) did not contain a marker which would allow positive selection in *D. discoideum*. Stable *D. discoideum* transformants carrying these plasmids are $lacZ^+$, indicating that insertion of the operator fragment at the three different positions in front of the suppressor tRNA gene does not interfere with tRNA gene expression.

Cotransformation of pGTETR+1 and pGTETR−7 with the Tet repressor, which encodes plasmid pDneoA6PTR, resulted in *lacZ* mutant strains. However, when these strains were grown in the presence of 30 μg of tetracycline per ml, active β-galactosidase was produced (Table 1). These results are consistent with the idea that in the absence of tetracycline the Tet repressor binds to its target sequence in front of the suppressor tRNA genes, thus interfering with tRNA gene transcription. In the presence of the specific inducer tetracycline, the Tet repressor loses affinity for its operator and tRNA gene transcription can occur, thus rendering *lacZ* mutant cells $lacZ^+$.

While selected clones carrying a suppressor tRNA gene with the operator inserted at position +1 were absolutely tight and strongly inducible, cells carrying a suppressor tRNA gene with the operator inserted at position −7 were slightly leaky when grown without tetracycline but still strongly inducible when tetracycline was added (Table 1).

A different result was observed when pGTETR−46 was cotransformed with plasmid pDneoA6PTR. The resulting transformants exhibited a $lacZ^+$ phenotype, regardless of whether the cells were grown with or without tetracycline (Table 1). Apparently, Tet repressor binding occurring on a tRNA gene with the operator insertion at position −46 is too far upstream to interfere with RNA polIII-catalyzed transcription. Differences in *lacZ* activity in individual transformants (Table 1) were due to the degree of plasmid amplification (data not shown) and probably to the site of integration.

Regulation by tetracycline occurs at the level of $tRNA^{Glu}$(Am) gene transcription. It is presumed that induction of tRNA suppressor expression accounts for the inducible expression of LacZ activity. To test this hypothesis, tRNA expression was analyzed in transgenic *D. discoideum* strains carrying a suppressor tRNA gene with *tetO* inserted either at position +1 or at position −7. Bulk tRNA was isolated from these strains before and 4, 8, and 24 h after addition of 30 μg of tetracycline per ml to the growth medium. These tRNAs were analyzed by primer extension with oligonucleotide DD8 (5′-CCAGTGTTAGAGAC-3′), which under our assay conditions recognizes exclusively

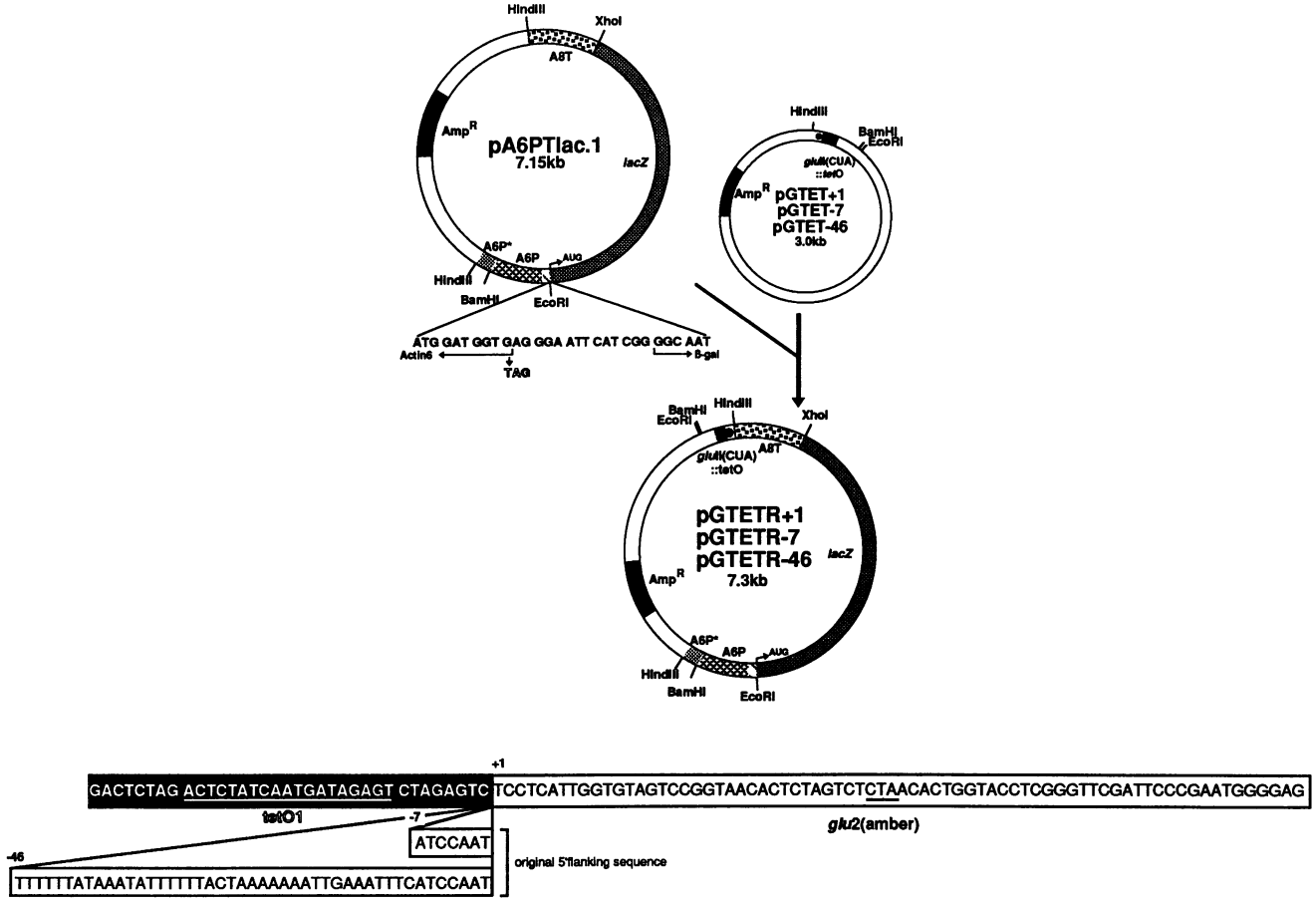

FIG. 3. Construction of plasmids pGTETR+1, pGTETR−7, and pGTETR−46. The *Hin*dIII fragment from pA6PTlac.1, containing the bacterial *lacZ* gene under transcriptional control of the *D. discoideum* actin 6 promoter (A6P) and the actin 8 terminator (A8T), was inserted into the unique *Hin*dIII site of either pGTET+1, pGTET−7, or pGTET−46 (see Materials and Methods).

suppressor tRNAs. Bound primers were elongated towards the 5′ end of suppressor tRNAs with reverse transcriptase, and cDNAs were visualized by autoradiography after electrophoretic size fractionation (Fig. 4). No cDNA was formed with primer DD8 on RNA prepared from untransformed Ax-2 cells. This underscores the specificity of the reaction, even in the presence of high levels of wild-type $tRNA^{Glu}$. Only marginal amounts of the suppressor were present in cells transformed with pGTETR+1 when these cells were grown without tetracycline. However, after 4 h of exposure to 30 μg of tetracycline per ml, significantly higher levels of suppressor tRNA were detected (Fig. 4). This concentration apparently represents steady-state levels, since the amount of suppressor tRNA did not rise substantially, even when cells were exposed to tetracycline for 24 h. All of the fragments resolved on the gel are specific, but incomplete cDNA fragments synthesized from amber suppressor tRNAs and their intensity reflect, to a first approximation, the amount of suppressor tRNA. These incomplete cDNA fragments occur because of strong stops of reverse transcriptase at transitions from the anticodon stem to the D stem (lower predominant bands) and at the transition from the D stem to the aminoacceptor stem (lower predominant bands) (16). A similar but not identical result was obtained when cells transformed with pGTETR−7 were analyzed. The tRNA was also induced by exposure to tetracycline, but in this case there was a higher level of suppressor tRNA under noninduced conditions (0 h). The slightly higher concentration of mature suppressor tRNA in the strain carrying the operator fragment at position −7 relative to the tRNA gene probably

TABLE 1. β-Galactosidase activity[a]

Clone	Mean enzyme activity (pkat/mg of protein) ± SD	
	−Tet	+Tet
+1/IV/A3	2 ± 2	90 ± 8
+1/IV/A2	2 ± 3	55 ± 7
−7/II/A1	7 ± 3	330 ± 46
−7/II/A3	6 ± 2	167 ± 20
−7/II/B1	5 ± 3	108 ± 12
−46/I/A2	9 ± 2	16 ± 2
−46/I/A3	6 ± 3	10 ± 3

[a] Cells were harvested from 100-ml logarithmically growing cultures either with (+Tet) or without (−Tet) 15 μg of tetracycline per ml. Extracts containing 75 to 150 μg of protein were analyzed for β-galactosidase activity with *o*-nitrophenyl-β-D-galactopyranoside as the substrate. The values represent at least three independent experiments. The low β-galactosidase activities in strains −46/I/A2 and −46/I/A3 were due to extreme instability of the original transformants, which expressed much higher activities in media with and without tetracycline shortly after the strains were established.

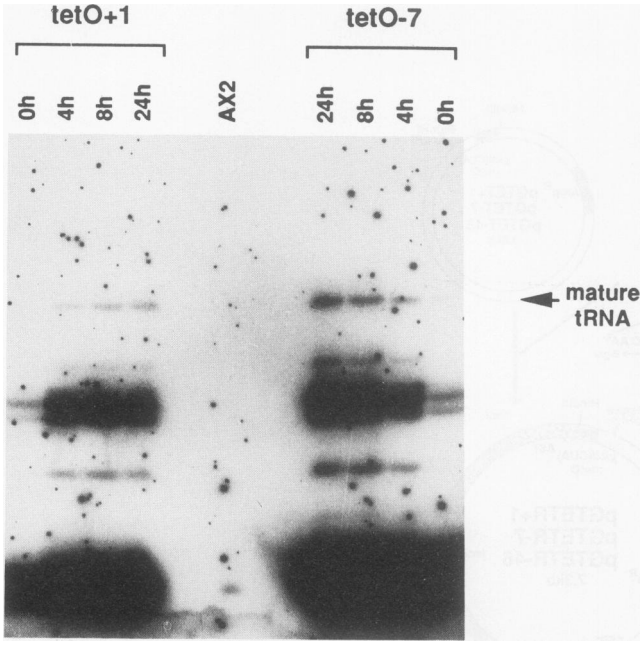

FIG. 4. Primer extension analysis of tRNAs isolated from untransformed Ax-2 cells (lane 5) and from transgenic *D. discoideum* strains carrying inducible suppressor tRNAs with operator insertions at positions +1 (lanes 1 to 4) and −7 (lanes 6 to 9). Samples were analyzed before (0 h) and 4, 8, and 24 h after addition of tetracycline to the cells.

explains the higher β-galactosidase activity measured for this strain in the absence of tetracycline (Table 1).

This analysis indicates the importance of an optimal position of the operator fragment relative to the mature tRNA coding region for optimal repression. It also indirectly provides an explanation for the severe leakiness of the pGTETR−46 transformants. In this construct, Tet repressor binding apparently occurs too far from the tRNA gene to allow efficient interference with tRNA gene transcription.

Kinetics of induction and deinduction of a polII gene product by controlled expression of tRNA^{Glu}(Am). Controlled expression of a suppressor tRNA offers the ability to regulate the synthesis of any polII gene product, provided the polII gene contains an internal nonsense codon. With this in mind, it is important to determine the timing of the appearance or disappearance of β-galactosidase following a change of conditions. After addition of tetracycline to the growth media, suppressor tRNA was detected after 4 h of Tet induction (Fig. 4). As expected, there was a slight delay in enzyme accumulation, with accumulation of significant amounts of β-galactosidase between 4 and 8 h. Cells were harvested from 100-ml logarithmically growing cultures. They were reinoculated into HL-5 medium (10^6/ml), tetracycline was added to 30 μg/ml, and the cells were shaken at 180 rpm. Extracts were prepared at the time points indicated and analyzed for β-galactosidase activity with *o*-nitrophenyl-β-D-galactopyranoside as the substrate. Relative β-galactosidase activities (normalized to the fully induced activity in growing cells of 90 pkat/mg [see the footnote to Table 1]) in clone +1/IV/A3 at 0, 4, 8, and 24 h after tetracycline addition were 2 ± 2, 3 ± 2, 10 ± 3, and 100 ± 3 pkat/mg, respectively. Enzyme activity reached a steady state within 24 h of Tet induction.

Replacement of media with phosphate buffer induces the developmental program in *D. discoideum*. Although it would be valuable to be able to regulate the expression of the tRNA suppressor during development, we were unable to check this possibility with the available constructs, since *lacZ* gene expression is controlled by the actin 6 promoter, which is known to become inactive early during development (43).

Repression of polII gene expression by depletion of the tetracycline in the culture is possible only for growing cells. This is due to the fact that tRNAs are very stable and appear not to be significantly degraded during the developmental cycle. In growing cells, two generations are required before the suppressor tRNA is diluted to a point at which phenotypic suppression is not observed any more. After that, the half-life of the particular protein under study determines its rate of disappearance. For β-galactosidase, 48 h of growth without tetracycline was required before basal levels were obtained (data not shown).

DISCUSSION

In this study, we provided a system which allows expression of a eukaryotic tRNA gene to be regulated without manipulation of the gene-internal promoter elements and thus alteration the structure of the tRNA itself. A target fragment of a prokaryotic DNA-binding protein was inserted into the 5′-flanking region of a suppressor tRNA gene. 5′-flanking regions of eukaryotic tRNA genes frequently exert a marked modulatory influence on tRNA gene transcription. This becomes apparent when the transcription efficiencies of different members of the same tRNA gene family are compared. Although these genes contain identical gene-internal control elements, they are transcribed with different efficiencies, apparently owing to their different 5′-flanking regions (2, 3, 10, 15, 24, 27, 37, 41, 47). For all of these examples, however, no indications exist that 5′-flanking regions can determine developmental or cell type-specific regulation of tRNA gene expression. In fact, regulation of tRNA gene expression has been demonstrated in only a very few cases (12, 34, 48, 50). Therefore, it is not surprising that systems which allow controlled transcription of eukaryotic tRNA genes are not available.

Regulation of the tRNA genes in this study is based on bacterial control elements acting in the 5′-flanking region of a eukaryotic tRNA gene, thus interfering with its transcription initiation. Different prokaryotic control elements, including the *lacZ* repressor-operator system (8, 29), the *lexA* system (6), and the *tet* repressor-operator system (22), have been used to control the expression of polII genes in eukaryotes. These studies proved that prokaryotic repressor proteins are able to enter a nucleus, even in the absence of a nuclear localization signal, and recognize their operator motifs, despite the very different chromatin structures of eukaryotic versus prokaryotic DNAs.

We used the tetracycline repressor-operator system to control a polIII gene. The *tetO1* fragment of the Tn*10*-encoded tetracycline resistance gene was inserted in front of an amber suppressor tRNA gene. Depending on the position of the operator, tRNA gene expression becomes tetracycline dependent in *D. discoideum* cells which also express the *tetR* repressor gene.

Despite the importance of the immediate 5′-flanking region for tRNA gene transcription, sequence requirements for this region are frequently surprisingly relaxed. This explains why the replacement of the natural 5′-flanking region with an oligonucleotide which serves as a sequence-specific target

for a well-characterized DNA-binding protein does not interfere with tRNA gene transcription per se. Only after the repressor is bound to its operator is transcription of the tRNA gene greatly reduced.

The position of the operator relative to the tRNA gene is crucial for tetracycline-sensitive regulation. The insertion has to be very close to the initiation nucleotide of the tRNA gene for its expression to be regulated. Insertion of the *tetO* fragment further upstream (i.e., at position −46) results in a constitutively expressed tRNA gene. Although the mode of regulation has not been rigorously determined, two explanations are most likely. Either the Tet repressor protein interacts with the RNA polIII complex such that it is inactivated, or the repressor protein masks the immediate 5′-flanking region where one important transcription factor binds (4, 32, 33) and where initiation has to take place (46).

Because nonsense suppressor tRNAs read their corresponding stop codons in almost any codon context, regulated tRNA genes may be used to control the expression of any class II gene, provided that those genes contain an internal translational stop signal. In such a system, nonsense mutations generally become conditional. Therefore, inducible suppressor tRNAs might become extremely useful tools in eukaryotic genetics. Since many tRNA genes can function in a wide host spectrum (9, 12, 13, 28, 30, 36, 52, 55), the system is certainly not restricted to *D. discoideum*. In fact, this has already been demonstrated by setting up tetracycline-dependent tRNA gene transcription in *Saccharomyces cerevisiae* (14). In organisms in which regulated polII promoters are not available, this type of suppressor tRNA gene may then be used as a master control gene to regulate the expression of any protein-encoding gene which carries a nonsense codon. Since the regulation described herein relies on a principle which is naturally realized exclusively in prokaryotes, induction and repression of the regulated tRNA gene do not induce pleiotropic effects. This is particularly true in *D. discoideum*, in which all natural termination appears to utilize the UAA ochre codon. Construction of this artificial suppressor system with an amber codon makes it unlikely that any endogenous genes will be affected by the presence of the suppressor tRNA.

The current alternative to the tRNA suppressor control system is to use a regulated promoter to control polII gene transcription. In *D. discoideum*, no nutritional, hormone, heat shock, or metal-inducible promoters have been characterized. Numerous developmentally regulated promoters are known, but they are not appropriate for the type of experiment envisioned for this control system. With developmental promoters, one cannot specifically control the expression of one particular gene in an otherwise unchanging environment. In addition, the cells cannot be maintained in an induced state for long periods of observation, owing to the rapid and terminal nature of the developmental program.

D. discoideum is becoming a popular system for analyzing the phenotype associated with mutations in genes that have a parallel function in mammalian systems. The basic architecture, signalling systems, and motility of the amoeba are very similar to those of mammalian tissue culture cells, yet *D. discoideum* is haploid and molecular genetic manipulation of genes through DNA transformation and high-frequency gene targeting is possible. Mutations in signal transduction proteins (35, 51), cytoskeletal proteins (11, 31), α-actinin (56), abp-120 (7, 9a), surface adhesion molecules (25), and many others have been made. When a mutant is isolated, the consequences can be either lethal, undetectable, or easily observed. In any of these situations, it is possible for secondary compensatory mutations to obscure the primary phenotype of lack of the protein. Owing to the long time between isolation of a mutant due to homologous recombination and analysis of the phenotype, it is possible that even when no phenotype is observed, subtle alterations could become masked by compensatory changes in the cell.

By using the tRNA suppressor system, it will be possible to isolate mutants by replacement of an endogenous gene with one containing a site-directed mutation that inserts a suppressible stop codon. During transformation and screening, strains can be kept in the presence of tetracycline to allow continued expression of the gene. Once the strain is identified, the tetracycline can be removed and the consequences of loss of the protein can be observed. This approach should eliminate the problem of phenotypic suppression by compensatory genetic or epigenetic alterations in the cell. Experiments are in progress in which a mutated copy of the endogenous *Dictyostelium* myosin heavy-chain gene is inserted into a regulated tRNA suppressor cell line.

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