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Regulated Expression of a Mammalian Nonsense Suppressor tRNA Gene In Vivo and In Vitro Using the *lac* Operator/Repressor System

DANIEL E. SYROID, RICHARD I. TAPPING, AND JOHN P. CAPONE*

Department of Biochemistry, McMaster University Health Sciences Centre, 1200 Main Street West, Hamilton, Ontario, Canada L8N 3Z5

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We have exploited the *Escherichia coli lac* operator/repressor system as a means to regulate the expression of a mammalian tRNA gene in vivo and in vitro. An oligonucleotide containing a *lac* operator (*lacO*) site was cloned immediately upstream of a human serine amber suppressor (Su^+) tRNA gene. Insertion of a single *lac* repressor binding site at position −1 or −32 relative to the coding region had no effect on the amount of functional tRNA made in vivo, as measured by suppression of a nonsense mutation in the *E. coli* chloramphenicol acetyltransferase gene following cotransfection of mammalian cells. Inclusion of a plasmid expressing the *lac* repressor in the transfections resulted in 75 to 98% inhibition of suppression activity of *lac* operator-linked tRNA genes but had no effect on expression of the wild-type gene. Inhibition could be quantitatively relieved with the allosteric inducer isopropylthio-β-D-galactoside (IPTG). Similarly, transcription in vitro of *lac* operator-linked tRNA genes in HeLa cell extracts was repressed in the presence of *lac* repressor, and this inhibition was reversible with IPTG. These results demonstrate that the bacterial *lac* operator/repressor system can be used to reversibly control the expression of mammalian genes that are transcribed by RNA polymerase III.

The development and application of a variety of inducible promoter systems has played an important role in the analysis of eukaryotic gene expression and function, particularly for those genes whose products are toxic to the cell. Promoters responsive to an assortment of inducing agents, including heavy metals, hormones, and heat shock, as well as several viral, cellular, and bacterial regulatory factors have been used with various degrees of success to manipulate gene expression in mammalian cells (23, 26–30, 34). These systems have been applied almost exclusively toward controlling the expression of genes that are transcribed by RNA polymerase II in which, for the most part, basal and regulatory promoter elements are found upstream of the transcription start site. The development of regulatable expression systems for genes that are transcribed by RNA polymerase III, which include the tRNA genes, is much less advanced. This situation stems largely from the fact that promoter elements for the majority of class III genes are intragenic, and thus their manipulation invariably results in alteration of the gene product itself (16, 42).

Our interest in regulatable class III promoters stems from our efforts directed toward the utilization of mammalian nonsense suppressor (Su^+) tRNA genes for the identification of nonsense mutations in essential viral and cellular genes. A number of suppressor tRNA genes have been constructed by site-directed mutagenesis of cloned tRNA genes and have been shown to efficiently suppress nonsense mutations in mammalian cells in transient transfection assays as well as in permanent cell lines (5, 6, 25, 35, 36, 41, 43). Constitutive high-level expression of Su^+ tRNAs in permanent cell lines, however, is deleterious to cell viability, and therefore a dependable method of controlling expression is required to generate stable suppressor cell lines (25, 41). One such approach makes use of an amber Su^+ $tRNA^{Ser}$ gene linked to a simian virus 40 origin of replication (41). Monkey cell lines containing integrated copies of this Su^+ tRNA gene as well as a thermosensitive simian virus 40 large-T-antigen gene afford a means of controlled temperature-dependent gene amplification, and consequently expression, of the resident tRNA genes. Cell lines utilizing this conditional amplification system have been used to identify and propagate several animal virus nonsense mutants; however, a number of disadvantages, including altered cell growth under conditions of suppression and restriction to cell types that are permissive for simian virus 40 replication, limit the general usefulness of this approach (41). In light of this finding, we have been attempting to develop alternate and more generally applicable methods of regulating Su^+ tRNA gene expression in vivo.

One potentially useful strategy involves using the bacterial *lac* operator/repressor system. The *lac* repressor is functional in mammalian cells, as demonstrated by its ability to inhibit transcription initiation as well as elongation of class II genes that contain appropriately placed *lac* operator (*lacO*) sites, presumably through steric hindrance or promoter occlusion (4, 8, 13, 21, 23). Repression can be relieved by the allosteric inducer isopropylthio-β-D-thiogalactoside (IPTG), which decreases the affinity of *lac* repressor for its operator (2). We reasoned that a similar approach could be used to regulate the expression of tRNA genes.

In this report, we demonstrate that expression of a human amber Su^+ tRNA gene, which contains an appropriately placed *lac* repressor binding site upstream of the coding region, can be efficiently repressed in vivo and in vitro with *lac* repressor and that this inhibition can be reversed with IPTG. These results provide the first example of a generally applicable system that affords the conditional in vivo and in vitro expression of a mammalian class III gene.

* Corresponding author. Bitnet electronic mail address: CAPONE[@]MCMASTER.CA.

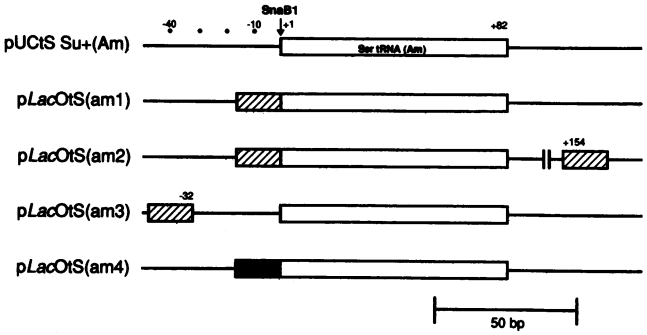

FIG. 1. Structures of *lacO*-containing Su^+ tRNA genes. The structure of the wild-type amber Su^+ tRNA is shown at the top. p*lacO*tS(am1) contains a single operator, 5'-ATTGTGAGCGCTCA CAAT, blunt-end ligated into the unique *Sna*BI site at −1. p*lacO*tS (am2) contains an additional operator site inserted into the unique *Sca*I site at +154. p*lacO*tS(am3) contains the operator cloned into a unique *Xba*I (GTCTAGAC) linker inserted at position −32. The oligonucleotide was identical to the one described above except that it was designed to contain *Xba*I-compatible ends. Sequences upstream from the *Xba*I site are vector derived. p*lacO*tS(am4) contains an extended operator sequence, 5'-TGTGGAATTGTGAGCGCTC ACAATTCCACA, cloned into the *Sna*BI site.

MATERIALS AND METHODS

Plasmids. The plasmids used in this study are diagrammed in Fig. 1. pUCtS-Su^+(am) contains a human amber Su^+ $tRNA^{Ser}$ gene and has been described previously (5). p*lacO*tS(am1) was constructed from pUCtS-Su^+(am) by inserting a double-stranded oligonucleotide containing a *lacO* sequence (5'-ATTGTGAGCGCTCACAAT-3') (4) into the unique *Sna*BI site immediately upstream of the coding region of the tRNA gene at position −1 by blunt-end ligation. p*lacO*tS(am2) was prepared from p*lacO*tS(am1) by inserting the *lacO* oligonucleotide into the unique *Sca*I site 154 nucleotides downstream from nucleotide +1 of the tRNA coding region. p*lacO*tS(am3) contains the *lacO* oligonucleotide 32 nucleotides upstream of the coding region. It was constructed from a derivative of pUCtS-Su^+(am) in which sequences upstream from position −32 were deleted and replaced with an *Xba*I linker GTCTAGAC (42a). The double-stranded oligonucleotide used in this plasmid was identical to the one described above except that it contained a four-base extension for direct ligation into the unique *Xba*I site. p*lacO*tS(am4) contains an extended *lacO* sequence (5'-TGTGGAATTGTGAGCGCTCACAATTCCACA-3') blunt-end ligated into the *Sna*BI site at position −1. This operator sequence has a higher affinity for *lac* repressor than does the wild-type *lac* operator sequence (40). pRSVIns expresses the *Escherichia coli lac* repressor, which has been modified by addition of the nuclear targeting signal from the simian virus 40 large T antigen to the carboxyl terminus (24). pRSV*cat*, which expresses the *E. coli* chloramphenicol acetyltransferase (*cat*) gene, and pRSV*cat*(am27), which contains a suppressible amber nonsense mutation in the *cat* gene, have been described previously (5, 17). Oligonucleotides were synthesized and purified through the Central Facility of the Molecular Biology and Biotechnology Institute, McMaster University.

Cell culture, DNA transfection, and CAT assay. All transfections were carried out by using BSC40 cells maintained at 37°C in Dulbecco's modified essential medium containing 10% calf serum. Transfections were performed by the calcium phosphate coprecipitation technique (18), using a total of 18 μg of DNA (made up with salmon sperm DNA as required) per 6-cm-diameter dish of cells at 50% confluency. Cells were shocked 4 h later with 10% dimethyl sulfoxide, upon which fresh medium containing 20 mM IPTG (where indicated) was added. Cells were harvested 48 h posttransfection, and CAT activity was determined, in the linear range of activity, by the liquid scintillation method as previously described and modified (12, 39). Plasmid DNA concentration was quantitated by fluorometry. Individual transfections were carried out in duplicate, and separate experiments were independently repeated two to three times except where indicated.

In vitro transcription. HeLa cell nuclear extracts were prepared and dialyzed against buffer D (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 20% [vol/vol] glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) as described previously (9). The final extract concentration was 7 to 10 mg/ml. The standard 20-μl transcription reaction mixture contained 80 to 100 μg of nuclear extract, 10 mM HEPES (pH 7.9), 50 mM KCl, 10% (vol/vol) glycerol, 8 mM creatine phosphate, 0.1 mM EDTA, 0.25 mM dithiothreitol, 2.5 mM $MgCl_2$, 0.4 mM each ATP, CTP, and UTP, 40 μM GTP, 2 μCi of [^{32}P]GTP (400 Ci/mmol), and 0.1 to 0.2 μg of template DNA; 30 to 50 ng of purified *E. coli lac* repressor (Stratagene) was added, in the presence or absence of 60 mM IPTG, to the reaction mixtures as indicated in the figure legends. The stock solution of *lac* repressor was 1 mg/ml in 20 mM Tris HCl (pH 7.4)–10 mM $MgCl_2$–5 mM β-mercaptoethanol–0.1 mM EDTA–500 mM NaCl–50% (vol/vol) glycerol. *lac* repressor protein was diluted in the same buffer. Control transcription reactions conducted in the absence of repressor protein were supplemented with the appropriate amount of *lac* repressor buffer. Transcription was allowed to proceed for 1.5 to 2 h, as indicated, at 30°C, and reactions were terminated by the addition of 10 vol of 0.3 M sodium acetate (pH 5.0)–0.5% sodium dodecyl sulfate followed by phenol-chloroform extraction and ethanol precipitation. Products were analyzed by electrophoresis on 10% polyacrylamide–7 M urea–1× TBE (89 mM Tris, 89 mM boric acid, 2.5 mM Na_2EDTA) gels followed by autoradiography. Autoradiographs were quantitated with a Hoefer scanning densitometer.

RESULTS AND DISCUSSION

***lac* repressor-mediated inhibition of tRNA gene expression in vivo.** tRNA gene promoters consist of two spatially separated intragenic sequence elements that in most instances are necessary and sufficient for transcription in vivo and in vitro (reviewed in references 16 and 42). These internal promoter elements are recognized by the general transcription factor TFIIIC, which initiates the ordered and cooperative assembly of a multicomponent transcription complex that includes transcription factor TFIIIB and RNA polymerase III. Extragenic sequences upstream of the coding region, while generally nonessential for transcription, can nevertheless influence the levels of expression by modulating the assembly or stability of the transcription complex (10, 19, 44).

Several studies using yeast tRNA genes have shown that TFIIIB, in cooperation with TFIIIC, is positioned upstream of the transcription start site and interacts with the 5' flanking DNA in a non-sequence-specific manner (3, 31, 32). We reasoned that the *E. coli lac* repressor, bound to *lac* operator sequences appropriately placed upstream of a

tRNA gene, might interfere with transcription, perhaps by preventing the formation of an active transcription complex, and thereby provide a means to control tRNA expression. To test this possibility, we inserted a *lac* operator-containing oligonucleotide into the upstream region of a human amber Su^+ $tRNA^{Ser}$ gene in plasmid pUCtS-Su^+(am). The functional expression of the Su^+ tRNA gene contained within this plasmid is easily monitored and quantitated in vivo following cotransfection with pRSV*cat*(am27), a CAT expression plasmid in which the *cat* gene contains a suppressible UAG nonsense codon (5).

p*lacO*tS(am1) (Fig. 1) contains a *lac* operator oligonucleotide inserted at position −1 at the unique *Sna*BI site, which precisely demarcates the 5′ flanking region and the coding region. Since the 18-residue-long oligonucleotide used in this construction is symmetrical, the relative center of the *lacO* site is positioned at −9. The effect of this insertion on expression of functional Su^+ tRNA was quantitated in vivo by cotransfection of BSC40 cells with pRSV*cat*(am27) and measuring CAT activity. As demonstrated in Fig. 2A, insertion of the *lac* operator sequence at this site had no significant effect on nonsense suppression activity in comparison with the wild-type tRNA gene. This relationship held over a wide range of tDNA concentrations (see Fig. 3) and is consistent with studies showing that while the 5′ flanking region is important for promoter activity of certain tRNA genes, there does not appear to be a strict sequence requirement (16, 42). To determine whether *lac* repressor can bind to this site in vivo and thus inhibit tRNA expression, cells were cotransfected with the *lac* repressor expression plasmid pRSVIns (24). As shown in Fig. 2A, inclusion of pRSVIns in the transfection resulted in a significant and selective inhibition of the level of nonsense suppression activity by p*lacO*tS(am1) but had no effect on suppression activity by the wild-type plasmid pUCtS-Su^+(am). The level of suppression was reduced approximately 75 to 85% for p*lacO*tS(am1) in the presence of pRSVIns, as determined from several independent transfections.

The plasmid vector for pUCtS-Su^+(am) and all of the other tRNA-containing plasmids is based on pUC (5). Thus, these constructs also contain a *lacO* site derived from the region of the *lac* operon that is present on this plasmid and which is situated approximately 500 nucleotides upstream from the tRNA coding region. As can be seen in Fig. 2A, *lac* repressor had no effect on suppression activity of the wild-type pUCtS-Su^+(am), demonstrating that this *lacO* site present in the plasmid backbone does not contribute to *lac* repressor-mediated inhibition. Similar results were obtained with use of a plasmid in which this naturally occurring *lacO* site was deleted (data not presented). The results demonstrate that *lac* repressor is capable of inhibiting the functional expression of a Su^+ tRNA gene only when the gene contains an appropriately placed *lacO* site.

Suppression activity can be allosterically regulated by IPTG. To determine whether *lac* repressor-dependent inhibition of p*lacO*tS(am1) tRNA expression could be reversed, CAT activity was measured in cotransfection assays carried out in the presence of 20 mM IPTG. As shown in Fig. 2A, IPTG quantitatively reversed repressor-dependent inhibition of Su^+ tRNA gene expression by p*lacO*ts(am1). IPTG on its own had no effect on expression of the wild-type gene or on p*lacO*tS(am1) (data not presented).

We next determined the effects of altering the position of the operator site relative to the tRNA gene or of using an operator site which has a higher affinity for *lac* repressor on repressor-mediated inhibition of expression. p*lacO*tS(am2),

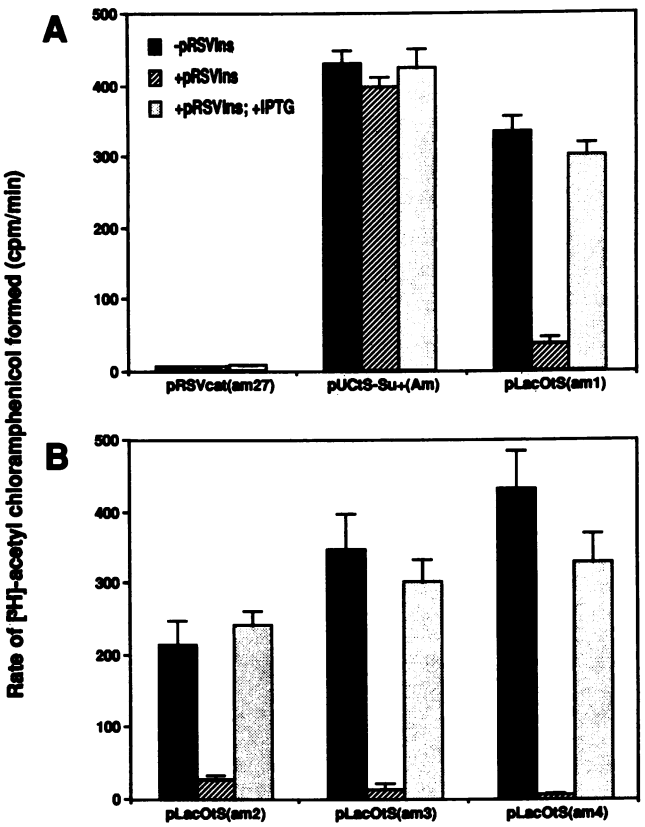

FIG. 2. Selective and reversible inhibition by *lac* repressor of phenotypic Su^+ tRNA expression in vivo of *lacO*-linked tRNA genes. (A) BSC40 cells (60-mm-diameter plates) were cotransfected in duplicate with 5 μg of pRSV*cat*(am27), 1 μg of Su^+ tRNA plasmids, and 1 μg of pRSVIns, as indicated. Where indicated, cells were incubated in the presence of 20 mM IPTG for the course of the transfection. CAT activity was determined 48 h later, using the scintillation counting method and measuring the rate of formation of ^{3}H-acetyl chloramphenicol from the slope of the line by linear regression analysis. The results are averages from three independent experiments (± standard error of the mean). (B) The various *lacO*-linked tRNA genes (0.25 μg) were transfected as described above. For p*lacO*tS(am3) and p*lacO*tS(am4), suppression activity was reduced by 95 to 98% by pRSVIns in comparison with the nonrepressed state. For p*lacO*tS(am2), activity was reduced by 75 to 80%. The results are averages (± range) from duplicate transfections. Corresponding symbols are as shown in panel A.

like p*lacO*tS(am1), contains the operator sequence inserted at the *Sna*BI site but also contains another operator sequence inserted downstream from the coding region of the tRNA gene at position +154, which is approximately 65 nucleotides downstream from the transcription termination site. As demonstrated by others, the *lac* repressor tetramer is able to bind cooperatively to spatially separated *lacO* sites, resulting in looping out of the intervening DNA and formation of a very stable protein-DNA complex (14, 20). The rationale for constructing p*lacO*tS(am2) was based on the supposition that cooperative binding of *lac* repressor and perhaps looping out of the tRNA gene might afford more stringent repression. Nonsense suppression activity from this plasmid was slightly reduced (~50%) compared with the wild-type gene or p*lacO*ts(am1) activity; however, there was no difference in the relative level of repression observed with

lac repressor or the extent of derepression in the presence of IPTG compared with results for p*lacO*tS(am1) (Fig. 2B).

To determine the effects of placing an operator further upstream, p*lacO*tS(am3), which contains the *lacO* site inserted 32 nucleotides upstream from the coding region, was constructed. Because of the presence of an *Xba*I linker at this site, this actually places the center of the operator site at position −47 relative to the coding sequence of the tRNA gene. As with p*lacO*tS(am1), the level of activity in the absence of *lac* repressor was not significantly affected by the insertion of the operator sequence at this site compared with the wild-type pUCtS-Su$^+$(am). Interestingly, *lac* repressor was able to inhibit Su$^+$ tRNA expression from p*lacO*tS(am3) and, in fact, did so slightly more efficiently than from p*lacO*tS(am1) (Fig. 2B). Activity was reduced approximately 90 to 95%, compared with the 75 to 85% reduction typically observed with p*lacO*tS(am1). Derepression by IPTG was approximately 85%. The results demonstrate that *lac* repressor bound to a site 32 nucleotides upstream from the coding region is still capable of effectively interfering with tRNA gene expression.

p*lacO*tS(am4) contains an extended, higher-affinity *lacO* site at position −1 compared with p*lacO*ts(am1), which positions the center of the operator sequence at position −15 relative to the coding region. Inhibition of suppression activity from this plasmid by *lac* repressor was also efficient, and under the standard assay conditions, expression could be inhibited almost completely (>98%), as shown in Fig. 2. Derepression with IPTG was approximately 75%. It is not known at present whether the more stringent repression observed with p*lacO*tS(am4) is due to the higher-affinity *lacO* site or due to its different placement relative to the tRNA coding region in comparison with p*lacO*tS(am1).

The efficiency of inhibition of transcription mediated by *lac* repressor in vivo is a function of the ratio of repressor molecules to DNA target sites and the dissociation rate of DNA-bound repressor (2). We therefore examined the effect of varying the amount of tDNA vis-à-vis pRSVIns on levels of nonsense suppression activity. Increasing amounts of p*lacO*tS(am1) were transfected into cells along with a constant amount of pRSVIns (1 μg) and pRSVcat(am27) (5 μg) in the presence or absence of 20 mM IPTG. Suppression activity was a linear function of tDNA concentration up to approximately 1 μg of transfected plasmid (Fig. 3A). In the presence of 1 μg of pRSVIns, the levels of activity were reduced 75 to 85% over a range of 0.05 to 1 μg of tDNA. This inhibition was quantitatively reversed in the presence of IPTG. With increasing ratios of p*lacO*tS(am1) to pRSVIns (5:1 by mass), however, *lac* repressor-dependent inhibition was not observed. This is likely the result of excess tDNA titrating out the available repressor molecules. These results demonstrate that the ratio of tRNA gene copy number to repressor protein is important for efficient inhibition and thus regulation of this gene.

The effect of increasing *lac* repressor concentration on tRNA expression was also determined. In this experiment, 0.25 μg of p*lacO*tS(am1) was cotransfected with pRSV-*cat*(am27) along with increasing amounts of pRSVIns in the presence or absence of IPTG. The maximum level of repression was observed when 0.25 μg of pRSVIns was used; increasing the *lac* repressor plasmid concentration beyond this point did not further inhibit Su$^+$ tRNA expression (Fig. 3B). The fact that suppression activity could not be repressed completely under these conditions may reflect a lag period between expression of the tRNA genes and the time required for the accumulation of sufficient repressor molecules to bind all of the target sites. However, this does not appear to be the case, since other *lacO*-linked tRNA genes, as described above, are more stringently repressed.

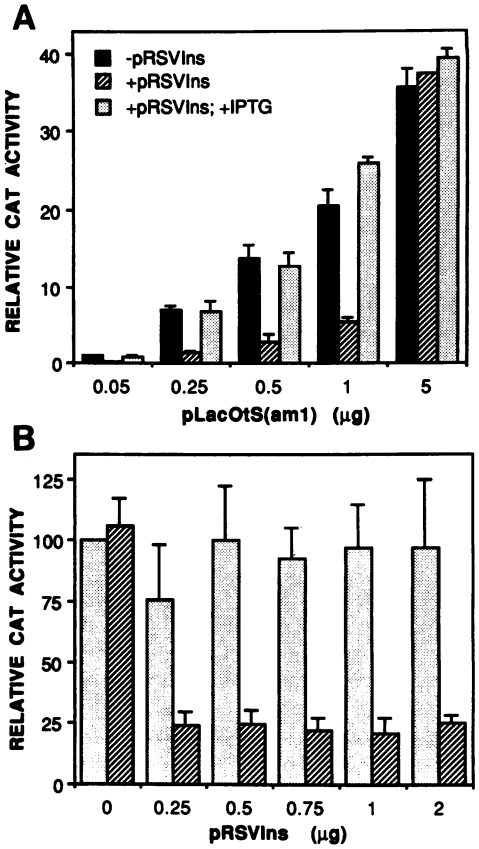

FIG. 3. Effects of increasing tRNA and *lacI* plasmid concentration on *lac* repressor-mediated inhibition of transcription. (A) BSC40 cells were transfected with 5 μg of pRSV*cat*(am27), 1 μg of pRSVIns, and various amounts of p*lacO*tS(am1) in the presence or absence of 20 mM IPTG, as indicated. CAT activity was determined as for Fig. 2. The results are averages from three independent experiments (± standard error of the mean) normalized against the CAT activity obtained from transfection of 0.05 μg of tDNA plasmid in the absence of pRSVIns, which was taken as 1. (B) Cells were cotransfected with 5 μg of pRSV*cat*(am27), 0.5 μg of p*lacO*tS(am1), and various amounts of pRSVIns, as indicated. The CAT activity was averaged from three independent experiments (± standard error of the mean) and normalized to the value for transfections carried out in the absence of pRSVIns, but in the presence of IPTG, which was taken as 100%. Corresponding symbols are as shown in panel A.

***lac* repressor inhibits tRNA gene transcription in vitro.** An obvious interpretation of the results presented above is that *lac* repressor inhibits nonsense suppression by blocking or interfering with transcription of the Su$^+$ tRNA gene. To test this possibility directly, the various tRNA genes were transcribed in vitro in HeLa cell nuclear extracts in the presence or absence of purified *lac* repressor. As shown in Fig. 4, the in vitro transcription activities of the various *lacO*-linked tRNA genes in the absence of exogenously added *lac* repressor were comparable to each other, varying ~ 10 to 30% in terms of overall template activity compared with the activity of the wild-type gene (compare lane a with lanes e, i, m, and q). The only exception was p*lacO*ts(am2), whose in vitro transcription activity was typically 30 to 50% of the levels

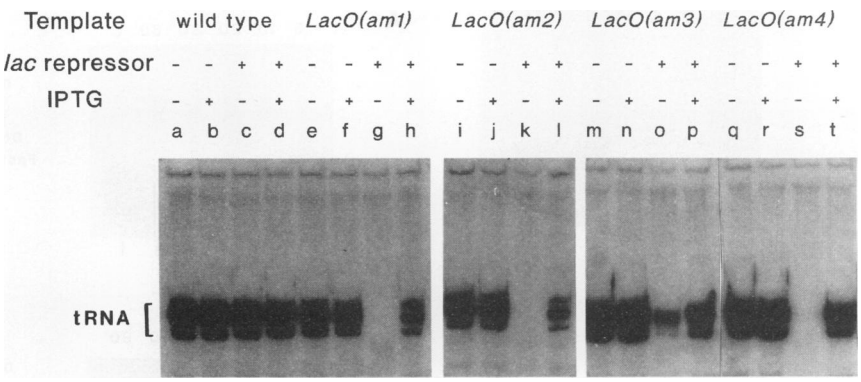

FIG. 4. Inhibition by *lac* repressor of transcription of *lacO*-linked tRNA genes in vitro. pUCtS-Su$^+$(am) (lanes a to d) or the various *lacO*-linked tRNA gene plasmids (lanes e to t) were transcribed in vitro, using HeLa cell nuclear extracts. Where indicated, the templates were preincubated with 50 ng of purified *lac* repressor (Stratagene) in the presence or absence of 60 mM IPTG for 10 min prior to addition of nuclear extract (factors) and NTPs. Transcription reactions were incubated for 2 h, analyzed by gel electrophoresis, and quantitated by densitometry. The figure is a composite of the same gel in which lanes i to l were exposed twice as long as the other lanes in order to normalize the intensity of the control lanes.

obtained with the wild-type gene. This finding is consistent with the in vivo results which demonstrated that the phenotypic expression of the Su$^+$ tRNA genes was not significantly affected by the extragenic insertion of the *lacO* sequence-containing oligonucleotides (Fig. 2).

Preincubation of the tRNA gene with purified *lac* repressor prior to the addition of nuclear extract and the ribonucleotide triphosphates (NTPs) resulted in a selective and stringent inhibition of transcription of the *lacO*-containing templates but had no effect on transcription directed by the wild-type tRNA gene (Fig. 4). Under the conditions used, 50 ng of *lac* repressor completely inhibited transcription of p*lacO*tS(am1), p*lacO*tS(am2), and p*lacO*tS(am4) (compare lanes e, i, and q with lanes g, k, and s). This finding suggests that once *lac* repressor is tightly bound to the DNA, it cannot be readily displaced by RNA polymerase III or other transcription factors. Under similar conditions, transcription of p*lacO*tS(am3), which contains a *lacO* site at position −32, was less efficiently inhibited by *lac* repressor (template activity was reduced approximately 50 to 70%) compared with the constructs containing a *lacO* site at position −1 (compare lanes m and o). Interestingly, *lac* repressor was capable of inhibiting the phenotypic expression of this gene in vivo as efficiently as did the other *lacO*-linked tRNA genes. The basis for this disparity is not yet known but may be related to differences in stability of the repressor-DNA complex in vivo compared with in vitro. *lac* repressor had no effect on transcription of the wild-type gene (lanes a and c) even when up to a 10-fold-higher concentration of repressor protein was used (data not presented).

In all cases, *lac* repressor-dependent inhibition of transcription was relieved through the addition of IPTG to the transcription reactions (Fig. 4, lanes h, l, p, and t), with the extent of derepression ranging from 40 to 90% compared with control transcriptions. IPTG on its own, in the absence of *lac* repressor, had no effect on transcription (lanes b, f, j, n, and r).

***lac* repressor inhibits transcription of tRNA genes assembled into stable preinitiation complexes.** The kinetics and factor requirements for establishing the formation of the preinitiation complex on class III genes have been established through a variety of template commitment, competition, and transcription inhibition experiments carried out both in crude extracts and with purified transcription factor components (15, 16, 33, 37). The preinitiation complex, which can assemble in the absence of NTPs, contains TFIIIB and TFIIIC and is refractory to challenge by a second template. In the presence of RNA polymerase III and NTPs, the transcription complex is stable through multiple rounds of transcription (37).

In the in vitro experiments described above, the tRNA^{Ser} gene was first preincubated with *lac* repressor in order to allow repressor to bind to its target site before addition of nuclear extract and NTPs. To further understand how *lac* repressor may prevent transcription of the tRNA gene, experiments were performed in which the template was first preincubated with nuclear extract in the absence of NTPs in order to allow stable preinitiation complex formation to occur. Repressor and NTPs were then added sequentially to the reaction. The results with p*lacO*tS(am1) used as the template are shown in Fig. 5. As already demonstrated in Fig. 4, when the template was preincubated with *lac* repressor prior to addition of extract and NTPs, transcription of p*lacO*tS(am1), but not of the wild-type gene, was prevented (Fig. 5; compare lanes c and d). When the tRNA template was preincubated with *lac* repressor and nuclear extract for 10 min prior to the addition of NTPs, transcription was also inhibited (lane f). This result suggests that *lac* repressor is capable of competing with the polymerase III transcription factors for the template or of displacing a factor(s) from the template. In lanes g and h, the template was preincubated with nuclear extract for 10 min in the absence of NTPs to first allow the formation of a stable preinitiation complex. The conditions of preincubation were predetermined to be those precluding the transcription of a challenge template added to the reaction at a later time (37) (not shown). Following this preincubation, *lac* repressor and the NTPs were added, and the extent of transcription was determined after an incubation period of 2 h. Interestingly, under these conditions, transcription was again completely inhibited (lanes g and h). Similar results were obtained if the preincubation period was as short as 5 min or extended up to 60 min prior to the addition of *lac* repressor and NTPs (Fig. 6A). These results demonstrate that *lac* repressor is capable of inhibiting transcription of tRNA genes that are already assembled into stable preinitiation complexes.

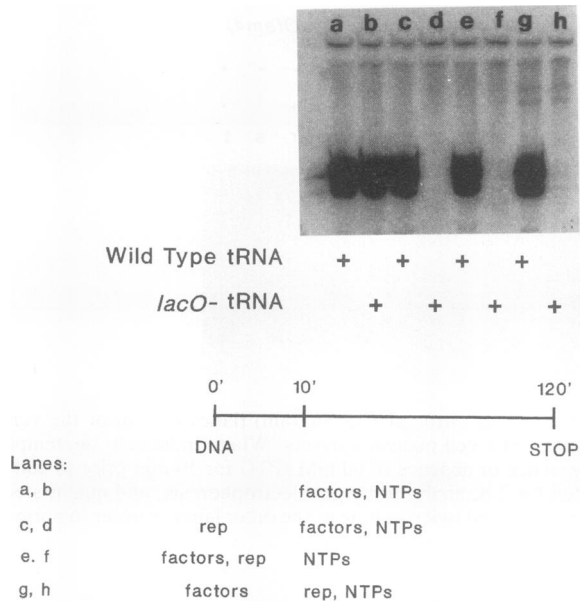

FIG. 5. Evidence that *lac* repressor prevents transcription of tRNA genes already assembled into a preinitiation complex. Preincubation reactions were performed as diagrammed at the bottom. Lanes a and b are control transcription reactions using wild-type pUCtS-Su$^+$(am) and p*lacO*tS(am1), respectively. In lanes c and d, templates (0.1 μg) were preincubated with *lac* repressor (50 ng) prior to the sequential addition of nuclear extract (factors) and NTPs. In lanes e and f, templates were preincubated with nuclear extract and *lac* repressor for 10 min prior to the addition of NTPs. In lanes g and h, templates were preincubated with nuclear extract for 10 min (a time sufficient to allow formation of the preinitiation complex) prior to the addition of *lac* repressor (50 ng) and NTPs. In this case, the NTPs were added 1 min after repressor addition. Reaction mixtures were incubated for 2 h following the addition of NTPs.

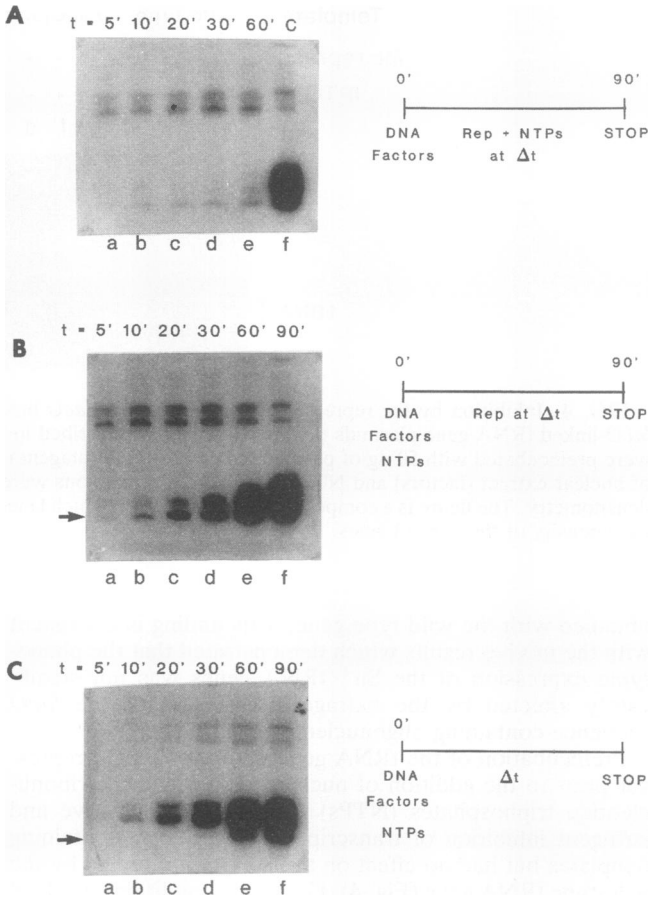

FIG. 6. Prevention of reinitiation of transcription by *lac* repressor. Transcription reactions were carried out as diagrammed on the right. In panel A, 0.2 μg of p*lacO*tS(am1) was incubated with nuclear extract for various times as indicated prior to the sequential addition of 50 ng of *lac* repressor (Rep) and the NTPs. There was a 1-min interval between the addition of repressor and NTPs. Reaction mixtures were incubated for 90 min following the addition of NTPs. Lane c of panel A is a 90-min control transcription reaction carried out in the absence of *lac* repressor. In panel B, the template was incubated with nuclear extract in the presence of NTPs. *lac* repressor was added at various times afterward as indicated. Reaction mixtures were incubated for 90 min from the time of addition of the NTPs. In panel C, template was incubated with nuclear extract and NTPs, and transcription was terminated at the times indicated with 10 volume of 0.3 M sodium acetate (pH 5.0) containing 0.5% SDS. The arrows correspond to the mature-size tRNA.

***lac* repressor prevents multiple rounds of transcription from actively transcribing complexes.** In Fig. 6, the effect of *lac* repressor on transcription of tRNA genes already assembled into actively transcribing complexes was examined. p*lacO*tS (am1) tRNA template was incubated with HeLa extract and NTPs for various times to allow initiation of transcription to occur prior to the addition of *lac* repressor (Fig. 6B). In each case, products were analyzed 90 min following the addition of NTPs. Transcription was reduced but not completely inhibited when *lac* repressor was added at any time following the addition of NTPs. The amount of product made was proportional to the time interval that had elapsed prior to *lac* repressor addition and, furthermore, coincided with control reactions that were carried out in parallel in the absence of *lac* repressor but that were prematurely terminated at the corresponding times (Fig. 6C). The results indicate that *lac* repressor is able to prevent multiple rounds of transcription of actively transcribing complexes, presumably by inhibiting reinitiation of transcription. It is interesting to note that while the overall amount of product made in Fig. 6B and C was comparable for each relative time point, the proportion of mature-size tRNA transcripts observed in Fig. 6B (arrow) was greater than in the corresponding control lanes in Fig. 6C. This result is expected since all reactions in the former case were carried out for 90 min, thereby allowing sufficient time for the processing and maturation of the primary transcripts compared with the prematurely terminated control reactions.

Conclusions. We have demonstrated that the *E. coli lac* repressor, bound to its cognate operator site appropriately placed upstream of a human tRNA gene, can be used to stringently and reversibly block expression of this class III gene both in vivo and in vitro. While this report was in preparation, Dingermann et al. (11) described the use of the bacterial tetracycline repressor/operator system to regulate the expression of an amber Su$^+$ tRNA^{Glu} gene in vivo in *Saccharomyces cerevisiae*. The work described by these investigators and that described herein demonstrate for the first time that prokaryotic regulatory elements can be effectively used to regulate the expression of genes that are transcribed by RNA polymerase III in both yeast and mammalian cells.

While the precise mechanism by which *lac* repressor

inhibits expression of the operator-linked tRNA gene is not yet known, a likely explanation is that the DNA-bound *lac* repressor prevents transcription by sterically interfering with the interaction of the transcription factors with the DNA or by preventing the proper assembly or function of the RNA polymerase III transcription complex. The importance of the 5′ flanking region in tRNA gene transcription has been documented in many cases, and it has been demonstrated that the RNA polymerase III transcription complex extends over the transcription start site and up to position −45 (7, 10, 19, 32, 44). For yeast tRNA genes, it has been established that the essential transcription factor TFIIIB, through interaction with TFIIIC, is positioned over the 5′ flanking region and interacts strongly with DNA in a non-sequence-specific manner (3, 31, 32). Thus, one plausible conclusion from our data is that *lac* repressor interferes with the proper assembly and function of TFIIIB, although it is not yet known whether mammalian TFIIIB behaves in a manner strictly analogous to that of its yeast counterpart. As an extension of our findings, it is interesting to speculate that tRNA gene expression may be regulated in vivo by gene-specific factors that selectively interact with 5′ flanking elements and that modulate the assembly or function of the transcription complex (16).

Our in vitro results show that *lac* repressor can completely block the expression of tRNA genes both before and after they are assembled into a preinitiation complex and can prevent multiple rounds of transcription of actively transcribing complexes. Thus, *lac* repressor can interfere with different stages of transcription, depending on the experimental conditions. These results suggest that the *lac* repressor protein may serve as a novel and useful reagent for examining mechanisms of transcription. For instance, by systematically varying the position of the *lacO* site proximal to the tRNA gene, one may be able to selectively and reversibly examine the different steps and requirements involved in preinitiation complex assembly, initiation, and perhaps elongation and termination of transcription.

lac repressor bound to an operator site placed at position −32 was still capable of inhibiting transcription in vivo and in vitro. As described above, this finding is consistent with DNA protection studies of yeast tRNA genes demonstrating the involvement of flanking sequences up to position −45 in protein-DNA interactions which may be important for transcription (32). We have yet to determine the most distal site at which the *lac* operator sequence may be placed and still afford *lac* repressor-dependent inhibition. Dingermann et al. (11) showed that in vivo expression in *S. cerevisiae* of a Su^+ $tRNA^{Glu}$ gene containing a tetracycline repressor binding site at position −46 was not inhibited by *tet* repressor, while *tet* repressor bound at position −7 completely inhibited transcription. Determining both the 5′ and 3′ extremities of *lacO* sequence placement that still allows repressor-mediated inhibition of transcription may provide a unique means of examining certain aspects of the spatial arrangement and constraints of transcription complexes that may not necessarily be related to the direct DNA binding of transcription factors and which are difficult to examine by more conventional strategies.

Steric hindrance is not the only mechanistic explanation for transcription inhibition by *lac* repressor. In *E. coli*, the *lac* repressor is able to inhibit RNA chain elongation through direct interactions with RNA polymerase itself in the transcribing complex, resulting in abortive RNA synthesis (38). While there is no evidence that *lac* repressor interacts directly with RNA polymerase III, it is interesting to note that there is some degree of evolutionary and functional conservation among bacterial and eukaryotic RNA polymerases (1, 16).

We have shown that under appropriate conditions, it is possible to efficiently and conditionally regulate Su^+ tRNA gene expression in vivo by using the *lac* repressor/operator system. *lac* repressor protein is functional in a wide variety of cell types and has been used to control the expression of integrated class II genes that contain suitably placed *lac* operator sequences (13, 22, 23, 34). Thus, exploiting this strategy for the allosteric regulation of Su^+ tRNA expression in vivo promises to be a feasible and generally applicable approach for the establishment of an assortment of stable mammalian suppressor cell lines. This procedure will facilitate the development of approaches directed toward the identification and characterization of nonsense mutations in viral and cellular genes that are more practical than the approaches currently available (41). Finally, in addition to controlling tRNA gene expression, the *lac* repressor/operator system should afford a potentially useful method of regulating and examining the expression of other genes that are transcribed by RNA polymerase III.

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