A TATA Element Is Required for tRNA Promoter Activity and Confers TATA-binding Protein Responsiveness in Drosophila Schneider-2 Cells*

(Received for publication, November 20, 1998, and in revised form, January 28, 1999)

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In contrast to yeast and mammalian systems, which depend principally on internal promoter elements for tRNA gene transcription, insect systems require additional upstream sequences. To understand the function of the upstream sequences, we have asked whether the Bombyx mori tRNAAla and tRNAG34 genes, which are absolutely dependent on these sequences in vitro, also require them for transcription.

We introduced wild-type and mutant versions of the Bombyx tRNAAla genes into Drosophila Schneider-2 cells and found that the tRNAAla gene is efficiently transcribed and that its transcription depends strongly on the distal segment of its upstream promoter. In contrast, the tRNAG34 gene is inefficiently transcribed, and this inefficiency results from lack of a specific sequence within the tRNAAla upstream promoter. This sequence, 5′-TTTATAT-3′, is sufficient to increase the activity of the tRNAAla promoter to that of the tRNAG34 promoter. Moreover, promoters containing the 5′-TTTATATAT-3′ element are stimulated by increased levels of cellular TATA-binding protein. Together these results indicate that, in insect cells, a TATA-like element is specifically required to form functional TATA-binding protein-containing complexes that promote efficient transcription of tRNA genes.

Transcription of cloned genes in cell-free extracts has been widely used to define the promoters of tRNA genes. This approach has established the contribution of gene-internmal promoter elements (reviewed in Refs. 1 and 2) and has revealed the effects of 5′-flanking sequences (both positive and negative) in a variety of organisms (2). In vivo assays to determine the biological relevance of the class III promoter elements defined in vitro have not been employed widely, but they suggest that at least some promoter elements identified in vitro are also required in vivo. For instance, the canonical A and B boxes function in vivo in yeast (3) and trypanosomes (4), and 5′-flanking sequences appear to modulate promoter function in several organisms (5–10).

There has been no systematic analysis of the function of upstream flanking sequences in vivo in any system however. Such an analysis of silkworm tRNAAla genes could be particularly informative because transcription of these genes in vitro by homologous transcription machinery is strongly dependent on upstream sequences (2). Moreover, the two AT-rich sequence blocks that provide most of the upstream promoter function for the silkworm tRNAAla also occur in other silkworm RNA polymerase III (pol III) templates (11–15). These sequences thus have the potential to comprise a class of general upstream promoter elements for polymerase III-dependent genes, something that has not been recognized in any other system (16–18). Upstream promoter elements are also responsible for the differential transcription in vitro of silk gland-specific (SG) and constitutive (C) silkworm tRNAAla genes (19).

To determine whether upstream sequences contribute to promoter function in vivo, we have transiently introduced wild-type and mutant tRNAAla genes into Drosophila Schneider-2 cells. Although they are heterologous, these cells can be transfigured efficiently (>10% (20)), and the upstream sequence dependence of the Drosophila pol III transcription machinery suggests functional similarity to the Bombyx machinery (reviewed in Refs. 2 and 21). In addition, because Drosophila S2 cells are not derived from silk glands, they provide an opportunity to ask whether C and SG upstream promoters are differentially active in non-silk gland cells as they are in non-silk gland extracts. In this paper we show that the activity of the tRNAAla upstream promoter in Drosophila S2 cells depends on a subset of the sequences required in vitro. Specifically, a TATA-like sequence located from positions −31 to −25 is the key element. Moreover, the wild-type C and SG upstream promoters are differentially active in vivo, and introduction of the TATA element raises SG promoter activity to the level of the C promoter. Overproduction of TBP in vivo stimulates promoter activity only if the TATA-like element is present. Thus, it is likely that in insect systems, the TATA-like element functions to facilitate the formation of active TBP-containing transcription complexes on tRNA genes.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The parental wild-type constitutively expressed tRNAAla gene used in this work (tRNAAla) contained sequences from −34 to +215 (with respect to the transcription initiation site) cloned into a derivative of pUC13 in which the HindIII site had been replaced by an MluI site (pUC13M). Mutant derivatives of the upstream promoter from −11 to −34 were made in portions spanning the “D,” “TAT,” “I,” and “AT” regions using oligonucleotide-directed mutagenesis as described previously (13). The wild-type silk gland-specific upstream promoter used here was fused to a fully wild-type tRNAAla

* This work was supported by United States Public Health Service Grants CA74138 (to D. L. J.) and GM25388 (to K. U. S.) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: pol III, RNA polymerase III; S2, Schneider-2; SG, tRNAAla; C, tRNAG34; CAT, chloramphenicol acetyltransferase; TBP, TATA-binding protein; PCR, polymerase chain reaction; bp, base pair(s).

‡ A. Trivedi, unpublished results.
gene coding and downstream region. It was derived from a previous chimera (SCC (19)) by using recombinant PCR (22) to convert the tRNA\textsubscript{Ala} start site (1 to 3 is AAC) to the tRNA\textsubscript{Ala} start site (1 to 3 is GTT). The chimeric tRNA\textsubscript{Ala}/tRNA\textsubscript{Ala} upstream promoter derivative in which the sequence \texttt{TTTTATAT} from the C promoter replaced the corresponding positions in the SG promoter was also constructed using recombinant PCR.

To distinguish the products of introduced genes from endogenous \textit{Drosophila} alanine tRNA, maxigene derivatives were made by cutting the genes at the unique internal \textit{Sph}I site and inserting a self-annealing, \textit{Xho}I-containing oligonucleotide (5\textquotesingle-GCTCGAGCCATG-3\textquotesingle) to yield maxigenes with 12 additional bp (Cwt maxi). An additional Cwt maxi +8 was made by restricting a Cwt maxi construct with \textit{Xho}I, filling in the \textit{Xho}I ends with the \textit{Klenow} fragment of DNA polymerase I, and re-ligating. To make antisense probes for RT\textsubscript{ase} protection assays, we cloned a Cwt maxi gene and a Cwt maxi +8 gene into \textit{pBluescript} (pSK\textsuperscript{+}), linearized these clones with \textit{Bam}HI, and used T7 RNA polymerase to generate antisense RNA (see below).

\textit{Transient Transfections—}Transient cotransfections were performed by a calcium phosphate precipitation technique (20). \textit{Drosophila} Schneider 2 cells were maintained at 25 °C in Schneider medium (Life Technologies, Inc.) containing 10% fetal bovine serum (Gemini Bioproducts). For transfections, cells were plated at 2.5 × 10\textsuperscript{6} to 5 × 10\textsuperscript{6} cells/25 mm\textsuperscript{2} in Corning brand tissue culture flasks. For each transfection, 6 µg of reporter plasmid DNA (tRNA\textsubscript{Ala} gene constructs) and 2 µg of a transfection efficiency control plasmid (pAct\textit{CAT} or pZIL, a luciferase-containing plasmid (23)) were used. The final DNA concentration was brought to 20 µg with \textit{pBluescriptSK} (pSK, Stratagene) or \textit{pUC} DNAs. All transfections were performed 3–6 times. Medium was changed 24 h post-transfection, and cells were harvested the following day. Approximately 2 × 10\textsuperscript{6} cells (one-fifth of the total cells) were used to make extracts to assay for chloramphenicol acetyltransferase or luciferase activity. Four-fifths of the cells (8 × 10\textsuperscript{6} cells) were used for RNA extraction to measure the transcription activity of the tRNA gene promoter.

\textit{Ribonuclease Protection Assay—}RNA was extracted using TriZOL (Life Technologies, Inc.) following the protocol provided by the vendor. The RNA yield was determined by measuring the absorbance at 260 nm. A ribonuclease protection assay was performed on the RNAs isolated from transiently transfected cells, using an \textit{RPA II} kit from Ambion. Briefly, a linearized plasmid containing the gene of interest served as a template for antisense transcript labeled with [\textit{α-32P}]\textit{CTP}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1}
\caption{The \textit{Bombyx} tRNA\textsubscript{Ala} upstream promoter is active in extracts of \textit{Drosophila} S2 cells. A, the sequence of a wild-type tRNA\textsubscript{Ala} gene from -12 to -34 is shown at the top, with the D, TAT, I, and AT regions of the upstream promoter delineated by brackets. The mutants are listed below, with the mutant sequences that have replaced wild-type sequences enclosed by boxes. Phenotypes are shown graphically and numerically at the right as means of percentages of the activity of a wild-type promoter determined in parallel. The size of 1 S.D. from each mean is shown. B, mutant phenotype is independent of template concentration. The transcription rates of the wild-type C upstream promoter (\textsuperscript{[\textcircled{C}]}) and the D region substitution mutant (\textsuperscript{[\textcircled{D}]}) are plotted as a function of the amount of template per reaction mixture.}
\end{figure}
tRNA Promoter Activity in Drosophila Cells

Fig. 2. Bombyx tRNA^{Ala} upstream promoter elements direct transcription in vivo in Drosophila S2 cells. A, RNase protection assays are specific for the products of B. mori tRNA^{Ala} genes expressed in Drosophila S2 cells. Shown are the products of protection by RNA from cells transfected with the 12-bp marked version of the Bombyx tRNA^{Ala} gene (Cut maxi), the 20-bp marked version of the Bombyx tRNA^{Ala} gene (Cut maxi +8), or pSK+ vector lacking a tRNA gene (−). Transfection efficiency was the same (±20%) in each case, as monitored by cotransfection with the actin-CAT plasmid described under “Experimental Procedures.” Products in the leftmost lane were derived by protection of an antisense “maxi” probe; products in the rightmost two lanes were derived by protection of an antisense “maxi +8” probe. Smaller fragments that represent protection of endogenous Drosophila tRNA^{Ala} were near the bottom of the gel and are not shown. B, RNase protection assays show the in vivo phenotypes of low resolution (upper panel) and high resolution (lower panel) mutants of the Bombyx tRNA^{Ala} upstream promoter. The transfection efficiency for each mutant, relative to that of the wild-type promoter, is shown below the lane. C, quantitative data for the mutants shown in B. The size of 1 S.D. from each mean is shown. Symbols are defined in the legend to Fig. 1.

(UCN, specific activity, >600 Ci/mmol), using the Maxiscript kit (Ambion). DNA was transcribed with T7 RNA polymerase, and the transcript was labeled with [α-32P]CTP (ICN, specific activity, >600 Ci/mmol). The probe was treated with DNase I to digest the template and was further purified by organic extractions and ethanol precipitation. Probe (0.5–1×106 to 1×106 cpm/reaction mixture) was hybridized with RNA (0.1–0.2 μg/reaction mixture) at 45°C overnight and then digested with RNase T1 (200 units/reaction mixture) and prepared for electrophoresis as described by the RPA kit manufacturer. Electrophoresis was on 8M urea-8% polyacrylamide gels, which were quantitated in two ways. 1) The gel was exposed to x-ray film for 30–120 min at −80°C, and the resulting autoradiographs were quantitated using a BioImage Scanner, or 2) the gel was exposed directly to a phosphor screen (Molecular Dynamics) for 60–120 min, and the data were collected using a Model 860 STORM PhosphorImager and quantitated using Image Quant software (Molecular Dynamics).

CAT Assay—The transfected cells were harvested and centrifuged, and the cell pellets (2×10⁶ cells) were resuspended in 90 μl of 0.25 M Tris, pH 7.5, followed by repeated freeze-thaw cycles. The extracts were then diluted 1:50 to 1:500 in 0.25 M Tris, and 5 μl of the diluted extracts were used to assay for CAT activity. The assay was performed as described previously (24). Products were analyzed by thin layer chromatography (TLC) and quantitated by scanning the autoradiograms in a BioImage Scanner.

Luciferase Assay—The transfected cells were harvested and centrifuged, and the cell pellets (2×10⁶ cells) were resuspended in 1× cell lysis buffer (Promega) and assayed with the luciferase assay system (Promega) using a Flow Tech Model 3010 Luminometer.
RESULTS

The Distal Segment of the Silkworm tRNA\textsubscript{Ala} Upstream Promoter Is Critical for Transcription in Drosophila Extracts—

To determine whether the tRNA\textsubscript{Ala} gene upstream promoter elements are recognized by the Drosophila transcription machinery, we first asked whether tRNA\textsubscript{Ala} transcription depends on these elements in vitro in Drosophila extracts. In these and subsequent experiments we used a set of mutants that systematically dissect the region between −15 and −34 of the tRNA\textsubscript{Ala} gene. Within this region, two AT-rich sequences, “AT,” located at −15 to −20 relative to the transcription initiation site, and “TAT” at −25 to −29 had previously been shown to be important for transcription in silk gland extracts (13). The sequence between TAT and AT (−21 to −24) is designated “T” (Intermediate), and the sequence upstream of TAT (−30 to −34) is designated “D” (Distal). In all cases, the mutant sequences that replace the wild-type sequences are GC-rich and do not alter the spacing of any presumptive promoter elements relative to one another or to the transcription initiation site.

The series of mutants was designed to delineate promoter function with increasing resolution, starting with a mutant that alters most of the upstream promoter. Substitutions in the three contiguous sequence blocks, TAT, I, and AT, eliminate promoter activity in Drosophila extracts. Two of the mutants with substitutions in two adjoining blocks of sequence (D-TAT or TAT-I) eliminate transcription entirely, whereas the third such mutant (I-AT) reduces promoter activity by only −30% (Fig. 1A). These results contrast with those observed in Bombyx extracts in which mutation of D-TAT, TAT-I, and I-AT yields promoters with −40, −5, and 2% wild-type activity, respectively. In the Drosophila system, mutation of the TAT region alone abolishes transcriptional activity (Fig. 1A), and mutation of the distal D region, by itself, reduces promoter activity 5-fold. In contrast, mutation of the AT region alone causes only about a 2-fold reduction. The observed mutant phenotypes were independent of DNA concentration (Fig. 1B), and variations in template concentration did not reveal additional mutant phenotypes. Taken together, these results suggest that in the Drosophila system, promoter function is largely confined to the distal part of the promoter (TAT, plus its neighbors), whereas in the Bombyx system, it is distributed between the distal TAT and the proximal AT regions.

Transient Expression of the Bombyx tRNA\textsubscript{Ala} Gene in Drosophila S2 Cells—For promoter analysis in vivo, we constructed derivatives of wild-type and mutant tRNA\textsubscript{Ala} genes that were marked with unique internal sequences (described under “Experimental Procedures”) to distinguish their transcripts from those of endogenous tRNA genes. The introduced sequences had no effect on the transcriptional activity of the genes in vitro in either Bombyx or Drosophila extracts.\textsuperscript{2} Drosophila S2 cells were cotransfected with the marked derivative of either a wild-type or a mutant tRNA\textsubscript{Ala} gene, together with a transfection efficiency standard in the form of a CAT reporter driven by the Drosophila actin 5C promoter (28). As shown in Fig. 2A, when the wild-type tRNA\textsubscript{Ala} gene marked by a 12-base pair insertion (Cwt maxi) was introduced into S2 cells, two new transcripts of −110 and 90 nucleotides were detected. To verify the identity of the protected RNAs, we introduced a different tRNA\textsubscript{Ala} maxi-gene derivative containing a longer inserted sequence (20 bp instead of 12 bp) and showed that the two transcripts migrated more slowly, as expected for the addition of 8 nucleotides.

The Distal Segment of the tRNA\textsubscript{Ala} Upstream Promoter Is Important in Vivo—The set of promoter mutants that had been tested in vitro (Fig. 1A) was introduced into Drosophila S2 cells. Fig. 2B shows the primary data from representative assays, and Fig. 2C shows the quantitative results, based on

\textsuperscript{2} Data not shown.
averages of at least three independent experiments. Simultaneous loss of the three sequence blocks, TAT, I, and AT, reduces promoter activity nearly 20-fold. Loss of only two regions is less deleterious. Removal of either the D-TAT or the TAT-I region reduces transcriptional activity 3- to 5-fold, and removal of the I-AT region has no detectable effect. The impact of losing individual smaller regions is more modest. As shown in Fig. 2C, mutation of the TAT region has the largest effect, reducing transcription 2-fold (to 44% of the wild-type level). Mutation of any of the other short sequence blocks has a smaller effect or none at all.

**tRNA^{Ala}_{Ala} and tRNA^{Ala}_{SG} Genes Are Differentially Transcribed in Vitro and in Vivo**—To compare tRNA^{Ala}_{Ala} and tRNA^{Ala}_{SG} promoter activity in vitro, we used constructs that yield identical tRNA^{Ala}_{Ala} primary transcripts in order to avoid differences in transcript processing or stability. The two promoters were first tested in vitro in extracts from S2 cells. Fig. 3A shows that the tRNA^{Ala}_{SG} promoter is at least 100-fold less active than the tRNA^{Ala}_{Ala} promoter, as expected from previous studies in other non-silk gland extracts. When these two promoters were tested in vivo, they also directed transcription with different efficiencies. As shown in Fig. 3B, the signal from the tRNA^{Ala}_{SG} promoter is 50-fold higher than that from the tRNA^{Ala}_{Ala} promoter. This difference was independent of the amount of template used over the range tested (2–8 μg).

What sequences in the upstream promoter are key discriminators between C and SG? The critical distal portion of the C promoter contains several overlapping AT-rich sequences that resemble binding sites for the TATA-binding protein. TATA boxes known to bind TBP are located in this position in pol II-transcribed genes (27) as well as in the pol III-transcribed gene, U6 (28). Interestingly, these TATA-like sequences are absent from the corresponding region of the SG promoter. Extensive point mutation indicates that the TATA-like sequence TTTATCT from –31 to –25 is the most effective of the distal AT-rich sequences for C promoter activity in vitro in Bombyx extracts. To ask whether this sequence could rescue the activity of the SG promoter in Drosophila cells, we constructed a chimeric C/SG promoter that placed the sequence in the corresponding position (–31 to –25) in the SG promoter (Fig. 4A). The T at position –24 in the SG promoter was mutated to a C to prevent the fortuitous introduction of additional TATA-like sequences. The data in Fig. 4B show that the promoter activity of this chimera is indistinguishable from that of the wild-type C promoter, both in vitro and in vivo.

**Overexpression of Drosophila TBP Differentially Affects tRNA^{Ala}_{Ala} and tRNA^{Ala}_{SG} Upstream Promoters**—The analysis of mutant tRNA^{Ala}_{Ala} promoters, as well as the chimeric tRNA^{Ala}_{Ala}/tRNA^{Ala}_{SG} promoter, indicates the functional importance of the TATA sequence in vitro and in vivo. Since this sequence resembles an optimal TBP binding site (29), and since TBP is required for transcription of tRNA genes in this and other systems (28), a plausible role for the sequence is to provide the C promoter with DNA contacts for TBP. Lack of the sequence in the wild-type SG promoter might prevent proper interaction

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with TBP. To test this idea, we performed transient transfections in an S2 cell line that was stably transformed with epitope-tagged *Drosophila* TBP under the control of a metallothionein promoter (26). Previous experiments have demonstrated that TBP is limiting for class III promoter activity in this cell type (26). As shown in Fig. 5, the wild-type C and SG promoters respond differently to TBP overproduction. TBP concentrations sufficient to increase the activity of the C promoter −2.5-fold do not stimulate the SG promoter. In contrast, the chimeric TATA-containing SG promoter responds to increased TBP concentrations just as the wild-type C promoter does.

**DISCUSSION**

We have shown that sequences upstream of the *Bombyx* tRNA*Arg* gene are functional as promoter elements *in vivo* in *Drosophila* S2 cells. Within this region, the TAT segment (−29 to −25) is the most important short sequence. Although tRNA promoter function is similar *in vivo* and *in vitro*, there are differences in detail. For instance, the effect of mutating short (4–5 bp) promoter sequences is less pronounced *in vivo* than it is *in vitro*. This result could suggest that sequence-specific interactions between transcription factors and upstream DNA do not occur *in vivo*, but the 20-fold effect of a 15-base pair substitution argues that they do. We think it likely that differences in the relative concentrations of particular transcription factors account for the disparity between *in vitro* and *in vivo* data. For example, TFIIB, the transcription factor complex that binds the tRNA*Arg* upstream promoter *in vitro* (30), may be less concentrated in the extracts and fractions used *in vitro* than it is within cells. The phenotypic variation observed *in vitro* and *in vivo* in *Xenopus* systems has been attributed to differences in the concentrations of various transcription factors (31–33). Moreover, quantitative Western blot analysis has shown directly that the components of yeast TFIIB are at least 100-fold more concentrated *in vivo* than they are in typical yeast transcription extracts (23). If a similar relationship exists for *Drosophila* cells and extracts, efficient incorporation of TFIIB into transcription complexes could require a higher affinity TFIIB binding site *in vitro* than it does *in vivo*, and, therefore, depend more heavily on a complete set of protein-DNA contacts.

Certain promoter mutants are differentially impaired in the *Drosophila* and *Bombyx* systems (see Fig. 6). Since in *Drosophila* these mutants have similar effects *in vitro* and *in vivo*, the observed differences most likely reflect true functional divergence between the *Drosophila* and *Bombyx* transcription machineries. The most obvious example is the strong dependence of the *Drosophila* transcription machinery on the distal portion of the promoter that includes the TAT region. This contrasts with the dependence of the *Bombyx* machinery on sequences in both the distal and proximal portions of the promoter, in particular on the TAT and AT sequences.

The different predilections shown by the *Drosophila* and *Bombyx* systems in these experiments fit with previous indications that the *Drosophila* transcription machinery is particularly sensitive to mutation of *Drosophila* pol III templates at about −25 (a position equivalent to *Bombyx* TAT), but is relatively indifferent to mutation closer to the transcription initiation site. Specifically, alteration of the natural sequences between 30 and 20 bp upstream of the transcription start site reduces the capacity of three different *Drosophila* tRNA genes (tRNA*Arg* (34), tRNA*Val* (18), and tRNA*Ala* (16)) and a *Drosophila* 5 S RNA gene (35) to direct transcription in *Drosophila* extracts. Although these effects are pronounced, a common sequence motif responsible for the activity of the wild-type −30 to −20 regions is not apparent. It is striking, however, that part of the critical region of the *Drosophila* 5 S RNA gene (−31 to −27; TATAA) strongly resembles the *Bombyx* TAT region (−29 to −25; TATAT). Mutation of this sequence alone eliminates 5 S transcriptional activity (35). In contrast, mutation of the region equivalent in position to the AT element (−20 to −15)
has a much smaller effect (35). These results suggest that the transcriptional enhancement due to the TATA-like sequence in Bombyx tRNA genes may reflect a general mechanism used in insect cells to increase the activities of specific 5 S and tRNA genes.

The Drosophila S2 assay system revealed that the C and SG promoters are differentially active in vivo, as they are in vitro. The lack of the sequence TTATAT (−31 to −25) in the SG promoter appears to account for the activity difference, since addition of this sequence endows an otherwise inactive SG promoter with an activity indistinguishable from that of a wild-type C promoter. What function does this sequence provide? Preliminary results indicate that it could be bound by purified silkworm TBP. Does this TBP-DNA interaction contribute to C promoter activity? The geometry of yeast transcription complexes suggests that it could, since the preferred geometry for transcription complexes formed on the wild-type SUP4 tRNA Tyv gene is one that places the upstream edge of TBP near the base pair at −30 (36). By analogy, the location of the TTATAT sequence in the C promoter should allow it to be contacted by TBP whose position is established by protein-protein contacts within the transcription complex. Thus, the primary role of this sequence may be to supply the C promoter with DNA contacts that add to protein contacts to create a high affinity TBP binding site. Lack of a comparable sequence may enfeeble the SG promoter.

In contrast to our results, previous studies have indicated that specific TBP-DNA contact is not required for transcription of 5 S and tRNA genes by pol III. In all systems tested, TFIIIB recruitment to these templates does not occur through DNA binding alone but depends on protein-protein interactions with TFIIIC (28). Moreover, there is direct evidence in yeast that specific interaction of TBP with a TATA element does not make a major contribution to TFIIIB recruitment, since mutation of the DNA binding domain of TBP has no effect on 5 S and tRNA transcription (37). Thus our results provide new evidence that, at least in insect cells, direct contact of TBP with DNA through a TATA element may be necessary for productive association of TFIIIB with the promoter.

Acknowledgments—We thank Nancy Ahnert for technical assistance and the Institute of Molecular Biology DNA Sequencing Facility for sequence analysis.

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