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Genetics

Regulation of glycine tRNA gene expression in the posterior silk glands of the silkworm *Bombyx mori*

(transcriptional control/regulatory elements/gel retardation/footprinting/*in vitro* transcription)

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ABSTRACT **The glycine tRNA genes in silkworm *Bombyx mori* contain two regulatory regions upstream of the transcription start site as identified by direct transcription of 5′ deletion mutants, transcription competition, gel mobility shift assays, and footprinting. A positive regulatory region is present in the immediate 5′ flanking sequences of the four $tRNA_1^{Gly}$ clones studied. This region is essential for cell-free transcription in homologous extracts. A negative regulatory region is present farther upstream, and transcription competition experiments indicate its presence in three of the four clones examined.**

The posterior silk gland of the silkworm *Bombyx mori* is a highly specialized organ involved in the synthesis of enormous amounts of the silk protein fibroin. Fibroin is rich in glycine (43%), alanine (28%), serine (12%), and tyrosine (5%) (1). To gear the system for synthesis of large quantities of fibroin, there is a functional adaptation in the silk glands of the *B. mori* larvae (2). The tRNA population in the posterior silk gland becomes highly enriched for the tRNA species cognate to the predominant amino acids in fibroin (3–5). To understand how the posterior silk gland produces such high levels of defined tRNA species, we have undertaken the study of regulation of expression of glycine tRNA genes. No $tRNA_1^{Gly}$ species that are exclusive to the silk gland have been identified so far (6–8), unlike the silk gland-specific $tRNA^{Ala}$ (9, 10). It was therefore important to compare the relative transcriptional efficiencies of more than one $tRNA_1^{Gly}$ gene. In this paper, we report the presence of two regulatory regions for these $tRNA_1^{Gly}$ genes. An upstream positive regulatory region, within 40 base pairs (bp) of the transcription start site, is required for cell-free transcription of the clones studied. A negative regulatory element is present further upstream in three of the four clones examined.

MATERIALS AND METHODS

Construction of Deletion Mutants. The six $tRNA_1^{Gly}$ genes used in this study were a gift from A. Fournier. These clones were isolated from a *B. mori* genomic library in the phage λ vector Charon 4. We have subcloned one of these, $pBma_2$ (8) as an *Eco*RI–*Sau*3A1 fragment in pUC18, which was designated pR8. The deletion mutants were constructed from this subclone. The 5′ deletion constructs include pX3 (−2 to +108), pSX (−40 to +108), and pKX (−150 to +108). The subclone pA3 has all of the upstream sequences of pR8 (−300) and the coding region up to the *Sma* I site (+53). These constructs are listed in Fig. 1*B*.

Preparation of Nuclear Extracts and *in Vitro* Transcription. Nuclear extracts from posterior silk glands of the fifth instar larvae were prepared as described for HeLa cell extracts (11) with modifications. The glands were homogenized in a buffer containing 2 M sucrose, 10% (vol/vol) glycerol, 10 mM Hepes (pH 7.9), 15 mM KCl, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. The homogenate was layered on a 3-ml cushion of the same buffer and centrifuged at 26,000 rpm for 1 hr at 4°C in a Beckman SW-41 Ti rotor. The pelleted nuclei were resuspended in a buffer containing 0.42 M NaCl, and the extracted proteins were dialyzed and processed as described (11).

In vitro transcriptions were done in a final volume of 25 μl containing 20 mM Hepes (pH 7.9); 60 mM KCl; 6 mM $MgCl_2$; 0.1 mM EDTA; 6 mM creatine phosphate; 50 μM each ATP, CTP, and UTP; 10 μM GTP; 5 μCi of [α-^{32}P]GTP (3000 Ci/mmol; 1 Ci = 37 GBq); 15 μl of nuclear extract; and 8 μg of supercoiled plasmid DNA per ml. After 1 hr at 30°C, reactions were terminated by the addition of 0.2% SDS, 10 mM EDTA, and 100 μg of tRNA carrier per ml. The RNA samples were run on 8% acrylamide/7 M urea gels.

Primer Extension and S1 Nuclease Analysis. Primer extension was done by the method of Carey *et al.* (12). The end-labeled primer (5×10^4 cpm) was hybridized to the *in vitro* transcribed RNA at 52°C for 8 hr. The hybridized primer was extended with avian reverse transcriptase at 42°C for 1 hr, and the products were extracted and run on 8% sequencing gels. The primer used was complementary to nucleotides +39 to +68 (8).

S1 nuclease analysis was done by the method of Favaloro *et al.* (13). The *in vitro* transcribed RNA from the clone pR8 was hybridized to three probes that covered regions −2 to +53, −40 to +53, and −300 to +53. The hybridized products were treated with S1 nuclease and run on 8% sequencing gels.

Gel Retardation Assays. For gel retardation assays, end-labeled fragments (10,000 cpm) were incubated in a 25-μl reaction mixture containing 12 mM Hepes (pH 7.9), 40 mM KCl, 5 mM $MgCl_2$, 4 mM Tris (pH 8.0), 0.6 mM EDTA, 0.6 mM dithiothreitol, 5% glycerol, 1 μg of double-stranded poly(dI-dC)·poly(dI-dC), and 1–3 μg of nuclear extract. The binding was allowed to proceed at 0°C for 15 min. After incubation, 5 μl of the loading buffer was added, and the samples were directly loaded on 6% polyacrylamide gels (acrylamide:methylenebisacrylamide ratio, 30:1) in TBE (45 mM Tris borate/12 mM EDTA) buffer of half strength. The gels were preelectrophoresed for 2 hr before the samples were loaded. The gels were dried and subjected to autoradiography.

Copper Phenanthroline Footprinting. Footprinting in solution was done as described by Kuwabara and Sigman (14). The binding reactions were treated with 2.5 μl of 1 mM *o*-phenanthroline/0.23 mM $CuSO_4$ and 2.5 μl of 58 mM mercaptopropionic acid for 5 min at 37°C, and the digestion was quenched with 2.5 μl of 28 mM 2,9-dimethyl-*o*-phenanthroline. The samples were then extracted with phenol, precipitated with ethanol, resuspended in 80% formamide, and loaded on an 8% sequencing gel.

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Abbreviation: bp, base pair(s).
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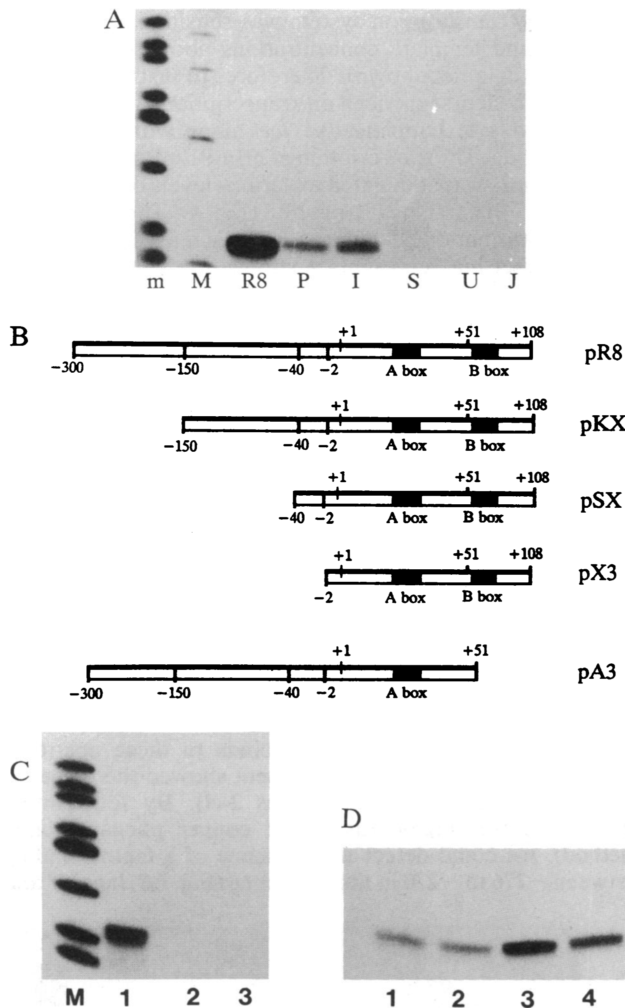

FIG. 1. *In vitro* transcription of *B. mori* $tRNA_1^{Gly}$ genes in nuclear extracts from the posterior silk gland. (*A*) The six different $tRNA_1^{Gly}$ genes used for transcription were pR8 (lane R8), pBmP1 (lane P), pBmI1 (lane I), pBmS1 (lane S), pBmU1 (lane U), and pBmJ1 (lane J). All clones were transcribed at a DNA concentration of 0.3 μg. Lanes M and m show pUC18 *Hin*fI and pUC18 *Hae* III digests, respectively. (*B*) Schematic representation of pR8 and its deletion derivatives. (*C*) Lanes: 1–3, transcription of pR8, pA3, and pX3, respectively, at 0.2 μg each; M, pUC18 *Hae* III digest. (*D*) Lanes: 1, pR8 at 0.2 μg; 2 and 3, pSX at 0.1 and 0.2 μg, respectively; 4, pKX at 0.2 μg.

RESULTS

Transcription of the $tRNA_1^{Gly}$ Genes in the Nuclear Extracts from the Posterior Silk Glands. The nuclear extracts from the posterior silk glands of *B. mori* larvae in the fifth instar could efficiently transcribe cloned $tRNA_1^{Gly}$ genes. Processing of the transcribed tRNA genes was also observed in these extracts. When transcription of six different $tRNA_1^{Gly}$ clones was carried out, a difference in the efficiencies of transcription of these clones was observed. The highest level of transcription was seen with clone pR8, followed by pBmI1 and pBmP1. One of these clones pBmS1 has two genes and gives rise to two distinct transcription products. The clones pBmU1 and pBmJ1 gave barely detectable levels of transcription (Fig. 1*A*). To identify the cis-acting elements required for the transcription of the $tRNA_1^{Gly}$ genes, deletion mutants were constructed from the clone pR8. The effect of the deletions was compared to the activity of the wild-type gene by transcribing the clones in homologous nuclear extracts. Deletion of the B promoter resulted in complete transcriptional inactivation of the gene (Fig. 1*C*, lane 2). The deletion of the entire upstream sequences also abolished transcription (Fig. 1*C*, lane 3). This suggested that, in addition to the internal promoters, upstream sequences are essential for the transcription of this gene. Interestingly, deletions upstream of −40 and −150 enhanced transcription, suggesting the possible presence of negative regulatory element(s) in that region (Fig. 1*D*, compare lanes 3 and 4 to lane 1).

Identification of the Transcription Initiation Site by Primer Extension and S1 Nuclease Analysis. To determine the initiation site of transcription, two $tRNA_1^{Gly}$ clones, pR8 and pBmP1, were used in primer extension assays. The sequence of the primer used corresponded to +39 to +68 of the coding region. The transcripts were found to initiate at −4 in the clone pR8 (Fig. 2, lane 3). A weak start at −3 and a strong site at −5 were found for pBmP1 (Fig. 2, lane 2). Since large amounts of mature $tRNA_1^{Gly}$ were present in the extracts, background hybridization of the primer to the endogenous transcripts in the absence of any added template also showed primer extension, but clearly up to +1 of the mature tRNA (Fig. 2, lane 1). *In vitro* transcripts of a $tRNA^{Glu}$ clone was used as a negative control to determine specificity of the primer. In this case, we did not see an extension product other than the signals picked up by the endogenous $tRNA_1^{Gly}$ extension (Fig. 2, lane 4).

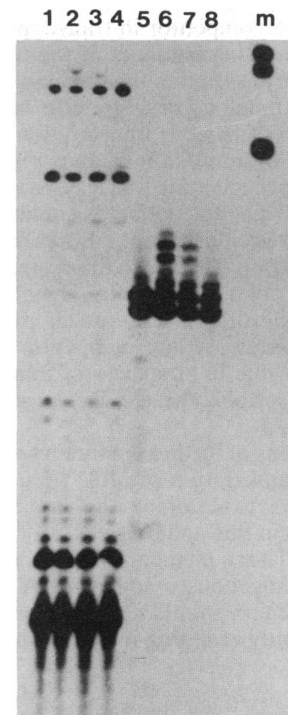

FIG. 2. Primer extension and S1 nuclease analysis. *In vitro* transcribed RNA from pR8 and pBmP1 was hybridized to an end-labeled primer and reverse-transcribed with avian reverse transcriptase. Lanes: 3, extension product of pR8 up to 72 nucleotides; 2, extension product of clone pBmP1 to 71 and 73 nucleotides; 4, extension product of the glutamic acid tRNA clone up to 68 nucleotides, which is the same as that caused by hybridization of mature tRNA (initiating at +1) endogenously present in the extracts (lane 1); m, pUC18 *Hin*fI digest. The DNA bands between the primer and the extended products represent positions at which reverse transcriptase extension stalled. For S1 nuclease analysis, the clone pR8 was *in vitro* transcribed and annealed to three probes. The probes from position −40 to +53 and from −300 to +53 showed S1 nuclease-protected bands of 57 nucleotides (lanes 6 and 7). The probe from −2 to +53 shows an S1 nuclease-protected band of 55 nucleotides (lane 8). The background hybridization due to mature tRNA present in the extracts, detected with the −300 to +53 probe (lane 5), shows a 53-nucleotide band. The weak signals observed above the 57-nucleotide band are due to incomplete S1 nuclease digestion.

These results were further confirmed by S1 nuclease analysis of transcripts from pR8 using three different probes: −2 to +53; −40 to +53; and −300 to +53. When the probes spanning −40 to +53 and −300 to +53 were used to detect the *de novo* transcribed RNA, a 57-nucleotide band corresponding to the unprocessed transcripts (initiating at −4) was detected (Fig. 2, lanes 6 and 7, respectively). Initiation at −4 was confirmed by using a probe, the 3′ end of which is at −2 of the gene. This probe detected a 55-nucleotide band, as expected, of a probe not containing the first two nucleotides of the unprocessed transcript (Fig. 2, lane 8). To determine the nature and extent of mature (processed) tRNA already present in the extract, a probe spanning positions −300 to +53 was used, and this detected the background as a 53-nucleotide product (Fig. 2, lane 5). This shows that unprocessed $tRNA_1^{Gly}$ levels endogenously present in the extracts are not readily detectable in this assay. These results are in agreement with the primer extension results.

Transcription Competition Assays. *In vitro* transcription of pR8 indicated that the upstream sequences up to −40 bp were required for transcriptional activity of this clone. To determine whether the requirement for 5′ upstream sequences existed for other $tRNA_1^{Gly}$ clones too, direct transcription competition assays were carried out. An oligonucleotide corresponding to the upstream sequence of pR8 from −5 to −38 was used as a competitor in transcription experiments along with subsaturating amounts of the template DNA. If there are specific transcription factors binding to this region, presence of additional copies of these sequences should result in inhibition of *in vitro* transcription. A drastic, concentration-dependent inhibition of transcription of pR8 was seen when this oligonucleotide was used as a competitor (Fig. 3*A*, lanes 2–4). This nucleotide sequence also inhibited transcription of other clones—i.e., pBmP1 (lanes 6 and 7) and pBmS1 (lanes 9 and 10)—although the upstream sequences of all of these clones are not identical. Lanes 1, 5, and 8 of Fig. 3*A* show the levels of transcription of pR8, pBmP1, and pBmS1 in the absence of any competitor DNA. That the inhibitions are not due to an excess of total DNA has been confirmed by transcribing these clones at a concentration of 0.3 μg (see Fig. 1*A*).

Since the deletions of further upstream sequences (beyond −40) enhanced transcription of pR8, we used transcription competition assays to examine the presence of negative regulatory regions in this and other $tRNA_1^{Gly}$ clones. If there is a negative regulatory element present in this region, the presence of an exogenously added DNA fragment corresponding to this region should compete for specific binding factors, consequently resulting in transcription stimulation.

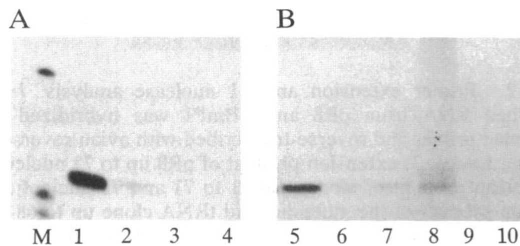

FIG. 3. Inhibition of *in vitro* transcription. *In vitro* transcriptions were done as described. In addition to the specific template (0.2 μg), a synthetic oligonucleotide corresponding to the −5 to −38 upstream sequence of pR8 was included as a competitor. Lanes: 1, 5, and 8, transcription product of the clones pR8, pBmP1, and pBmS1, respectively, in the absence of any added competitor; 2–4, extent of inhibition of transcription upon addition of increasing amounts (64, 128, and 192 ng) of the competitor; M, pUC18 *Hin*fI digest. Transcription of the clones pBmP1 and pBmS1 are also inhibited by 64 and 128 ng of the competitor (compare lanes 6 and 7 with 5, and compare lanes 9 and 10 with 8).

The *in vitro* transcription system was sensitive to the levels of DNA, and template concentrations above 0.3 μg were inhibitory (data not shown). Therefore, to test the effect of externally added sequences on transcription, we used suboptimal levels of template (0.1 μg) along with competitor DNA at 0.2 μg. The transcription of pBmP1, pBmS1, and pR8 but not pBmI1 was stimulated to various levels on addition of the −40 to −300 fragment from pR8 (Fig. 4). The stimulation observed was not due to a nonspecific increase in total DNA in the extract but represents a stimulation of transcription above that derived from 0.1 μg of specific template to levels greater than (pBmP1) or equal to that (pR8 and pBmS1) obtained with 0.3 μg of specific template (compare lane 2 with 3, lane 5 with 6, and lane 11 with 12 in Fig. 4). The same effect was not observed in the clone pBmI1.

Gel Retardation Assays and Footprinting. To detect the presence of any factors present in the nuclear extracts that could bind to the positive and negative regulatory regions, we used the fragments −40 to +53 (immediate upstream) and −40 to −300 (far upstream) separately for gel mobility shift assays and footprinting studies. Gel retardation with the −40 to +53 fragment of pR8 showed the presence of a single complex (Fig. 5*A*, lane 2 and Fig. 5*B*, lane 7) that could be chased effectively by an oligonucleotide spanning −5 to −38 of pR8 (Fig. 5*A*, lanes 3–5) but not by an oligonucleotide spanning +5 to +35 of pR8 (Fig. 5*B*, lanes 8–10). These results taken together with the transcription studies indicate that a positive regulatory factor binds to these upstream sequences. The −40 to −300 fragment showed the presence of two complexes (Fig. 6*A*, lanes 2–4). By footprinting analysis of this region (using the copper phenanthroline method), we could detect the presence of a factor binding between −276 to −270 in this sequence (Fig. 6*B*, lanes F and

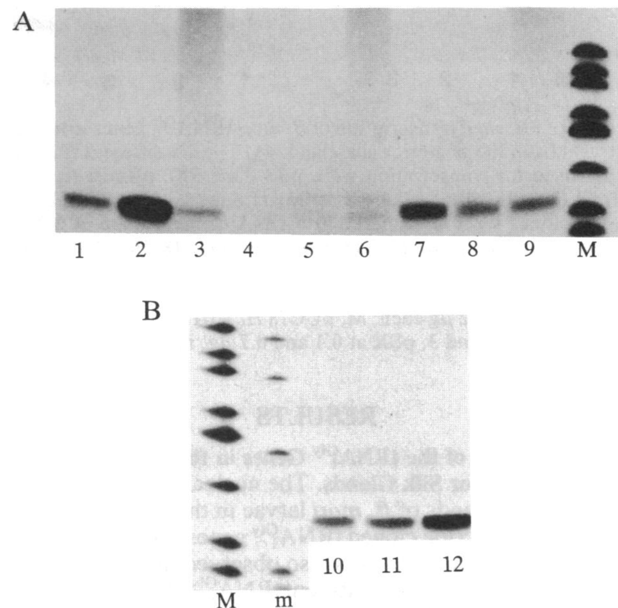

FIG. 4. Stimulation of *in vitro* transcription by the far upstream element. The cloned $tRNA_1^{Gly}$ genes were transcribed *in vitro* at a subsaturating concentration (0.1 μg) in the presence of the −40 to −300 fragment from pR8 as a competitor (0.2 μg). Lanes: 1, 4, 7, and 10, levels of transcription of the clones pBmP1, pBmS1, and pR8 in the absence of any competitor; 2, 5, and 12, levels of stimulation of transcription on addition of the competitor to the clones pBmP1, pBmS1, and pR8; 11, pR8 in the presence of a low concentration (64 ng) of competitor where stimulation is less evident (compare lane 11 with lane 12); 3, 6, and 9, controls for total DNA concentration effect (template at 0.3 μg); 8, level of transcription of clone pBmI1, which is not enhanced by the addition of competitor (compare lane 8 with 7); M and m, pUC18 *Hae* III and pUC18 *Hin*fI digests, respectively.

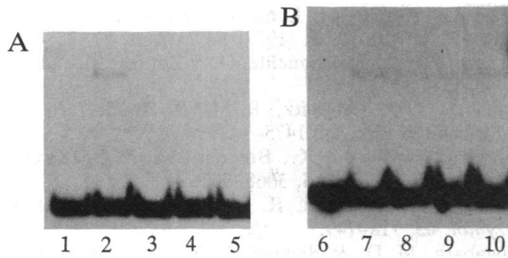

FIG. 5. Binding of factor to the immediate upstream region. The DNA fragment used in the gel retardation assays spanned −40 to +53 and was derived from pR8. The binding was done in the presence of competitor wherever indicated. (*A*) Lanes: 1, free DNA; 2, DNA with 1.5 μg of nuclear extract; 3–5, addition of competitor DNA spanning −5 to −38 at a concentration of 50, 100, and 150 ng, respectively. (*B*) Lanes: 6, free DNA; 7, DNA with 1.5 μg of nuclear extract; 8–10, DNA with 50, 100, and 150 ng of oligonucleotide spanning +5 to +35.

B). Although the signals obtained with free DNA alone in the region −270 to −276 were weak, the masking of signals was much more pronounced in the presence of protein binding. The presence of the footprint was consistent and reproducible. This demonstration of specific factors binding to this region has possible implications in the tissue-specific and developmental stage-specific expression of $tRNA^{Gly}_{1}$ genes in the posterior silk glands of *B. mori*.

DISCUSSION

We have focused our study on the transcriptional properties of four $tRNA^{Gly}_{1}$ genes. The clones used in this study vary in their 5′ flanking sequences (unpublished observations and ref. 15). The presence of a group of three sequences, TATAT, AATTTT, and TTC, within 30 bp upstream of the $tRNA^{Ala}$ gene and 5S RNA genes and 40 bp upstream of $tRNA^{Gly}_{1}$ genes has been reported and shown to be important for transcriptional activity (8, 16, 17). Unlike the $tRNA^{Ala}$ constitutive gene, the $tRNA^{Gly}_{1}$ gene (pBma2, corresponding to pR8 in this study), and the 5S RNA genes, all of which possess these three upstream sequences, the other three $tRNA^{Gly}_{1}$ clones used here have either one or two of the above mentioned nucleotide stretches. We report a difference in the transcription efficiencies of these clones. The clones pBmP1 and pBmI1 have only one of these upstream sequences (i.e., AATTTT) and are transcribed fairly efficiently, although at lower levels when compared with pR8, which has all three of these sequences. Therefore, it is clear that the 5′ flanking sequences are important for transcription and that at least one of these sequences are required for transcription. These results are further substantiated by the observation that the clone pX3, lacking the 5′ flanking sequences up to −2 (devoid of these three sequences) is not transcribed (Fig. 1*C*, lane 3). The presence of a factor binding to this positive regulatory element is apparent from the transcription competition and gel retardation assays. An oligonucleotide containing the three upstream motifs, when used as competitor, inhibits transcription of all $tRNA^{Gly}_{1}$ clones. The motif common to the clones pBmP1, pBmS1, pBmI1, and pR8 is the AATTTT block. Since transcription of all of the clones is inhibited, it is possible that the positive factor binds to this region. Deletion mutants of a 5S RNA gene lacking this AATTTT block have also been shown to be transcriptionally inactive (17). Hence, the importance of the 5′ flanking sequences in transcription of the $tRNA^{Gly}_{1}$ genes is evident.

The binding of the transcription factor TFIIIB occurs about 45 nucleotides upstream of the transcription initiation site in tRNA and 5S RNA genes of *Saccharomyces cerevisiae* (18–20). The upstream sequences have also been shown to be variable, and no conserved element for the binding of TFIIIB has been identified. We believe that the positive factor binding to the upstream sequences of all clones studied here could possibly be a general transcription factor like TFIIIB. Variability in the upstream sequences would result in a difference in the affinity of TFIIIB for these sequences and could result in a difference in the efficiency of transcription of these clones.

A suggestion that further upstream sequences (beyond −40), may have an important role in the regulation of a $tRNA^{Gly}_{1}$ gene has been made earlier based on *in vitro* transcription of a deletion mutant (8). We have tested this possibility directly with the same as well as other $tRNA^{Gly}_{1}$ clones, using this upstream fragment as a competitor. Since addition of the upstream fragment results in the stimulation of transcription of three clones pBmP1, pBmS1, and pR8, a similar element should be present in these clones. So far there are only a few reports on the presence of negative regulatory elements in the 5′ flanking sequences of tRNA genes. The *Xenopus laevis* $tRNA^{Met}_{1}$ gene contains two, alternating purine–pyrimidine stretches, TGCGCGTGC and ATGCACAGCGCA, which exhibit an inhibitory effect (21). The $tRNA^{Lys}_{2}$ genes of *Drosophila melanogaster* are also preceded by a GGCAGTTTTG tract, which inhibits transcription (22, 23). A similar tract, GGATTTTT, is found in the $tRNA^{Arg}_{1}$ genes of the same organism (24). By footprinting, we have identified the binding of a protein factor in the negative regulatory region of the $tRNA^{Gly}_{1}$ genes. This factor binds between −270 and −276 in the clone pR8, and this has the sequence TATATAA. Although it has been suggested that alternating purine–pyrimidine stretches can undergo conformational changes and thus provide a means of regulating expression (21), as yet we do not know how these alternating purine–pyrimidine tracts inhibit transcription. A stretch of alternating A-T residues has been shown to be less inhibitory than an alternating G-C tract of the same length (21). The effect of the negative regulatory element in *X. laevis* is also dependent on its location, and even a weak inhibitory sequence located between −32 and −42 can severely affect transcription when brought closer to the coding region. In *D. melanogaster* too, the inhibitory sequences are present between −23 and −13, which would again account for the poor transcription of this gene in homologous cell-free extracts. These two reasons—i.e., the presence of a weak inhibitory sequence of alternating A-T residues, and the far upstream location of the negative element in the $tRNA^{Gly}_{1}$ genes—could explain the efficient *in vitro* transcription of these genes in homologous nuclear extracts. This element perhaps is pres-

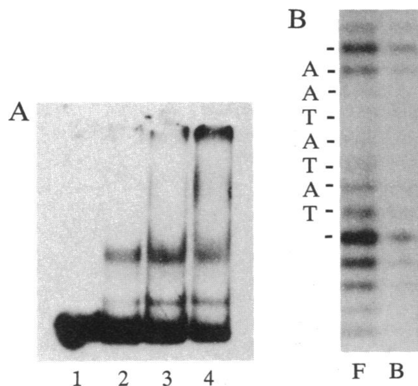

FIG. 6. (*A*) Gel retardation assay of the far upstream region. The DNA used spanned −40 to −300 and was derived from pR8. Lanes: 1, free DNA; 2–4, DNA with 1.0, 1.5, and 2.0 μg of nuclear extract. (*B*) Copper phenanthroline footprinting of the end-labeled upstream fragment (−40 to −300). Lanes: F, free DNA; B, DNA with 15 μg of nuclear extract.

ent closer to the coding region in the clone pBmP1, which would account for its higher stimulation in transcription competition assays. Progress towards understanding the regulation of expression of the $tRNA_1^{Gly}$ genes would be facilitated by purification and characterization of the proteins involved in this process.

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