Tandem Promoters in the Gene for Ribosomal Protein S20*

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Examination of the nucleotide sequence of the gene for ribosomal protein S20 (rpsT) of Escherichia coli suggested the presence of two promoters ("sites 1 and 2") separated by 90 base pairs (Mackie, G. A. (1981) J. Biol. Chem. 256, 8177-8182). We have investigated the properties of purified or cloned DNA fragments containing one or other or both these sites for their ability to promote transcription in vivo and in vitro. In reactions in vitro containing DNA and purified RNA polymerase as the sole macromolecular components, both sites 1 and 2 act as promoters directing the synthesis of "runoff" transcripts. The 5' terminal nucleotide of such transcripts have been determined by direct sequencing or by identification of the 5' terminal nucleoside 5'-triphosphate, 3'-monophosphate. In site 1, the major transcript initiates with GTP at residue 141 in the DNA sequence. A minor start occurs at residue 142 and uses CTP as the initiating nucleotide. In site 2, the major transcript (approximately 55% of all initiations in site 2) initiates with CTP at residue 232 while minor transcripts, each comprising approximately 20% of the total, initiate at residues 231 and 233 with GTP and CTP, respectively. In four methods of assay which reflect to varying extents the usage of promoters in vivo, site 1 is responsible for 10-30% of the total transcription of the gene for S20 and site 2 the remainder. Sites 1 and 2 appear to act independently and additively in assays based on the rate of synthesis of S20 in a system for coupled transcription and translation. Together, the two promoters for S20 are from 10-25-fold more active than the fully induced lac operon promoter.

The relative importance of transcriptional, as opposed to translational, controls in the regulation of the expression of ribosomal protein genes in Escherichia coli is still under debate (1-3). Five well characterized ribosomal protein promoters display no extensive mutual homologies in nucleotide sequence and exhibit no structural features easily reconciled with known patterns of ribosomal gene expression in vivo (4). It came as some surprise to find that the gene for ribosomal protein S20, rpsT, contains two promoter-like structures 42 and 132 residues to the 5' side of the initiation codon (5). These sites resemble bona fide promoters by homology with the conserved features typical of bacterial promoters (reviewed in Ref. 6) and by their ability to bind RNA polymerase in a manner which confers partial or complete protection from digestion with pancreatic DNase. One of the two putative promoters, site 1 in Ref. 5, differs from all other bacterial promoters so far described in that the apparent separation between the -10 and -35 regions (defined in Ref. 6) is only 14 bp whereas 17-19 bp is the norm (6). Indeed, deletions between -10 and -35 usually result in the loss of promoter activity (7, 8). In this work we have sought to apply a more rigorous definition of a promoter to the two sites adjacent to the gene for S20, namely to ask if either site 1 or 2 is capable of directing the initiation of transcription by RNA polymerase and, if so, at which residues the transcript(s) initiates. We have subsequently determined the relative efficiencies of the two sites at directing the transcription of the gene for S20 by several independent means.

Experimental Procedures

Strains and Media—Bacterial, plasmid, and phage strains have been described previously (5, 9) or below. The structures of plasmids and phages constructed for this work are illustrated schematically in Fig. 1. We used standard procedures for their propagation and for the purification of plasmids (10) or bacteriophages derived from M13 (11).

Digestion of DNA with Restriction Enzymes—All digestions were performed in a buffer containing 20 mM Tris-HCl, 5 mM MgCl2, and 1 mM dithiothreitol, pH 7.5. NaCl was added to 50 or 100 mM as required, as was Tris-HCl, pH 7.2, to 70 mM in the case of digestions with EcoRI. From 0.2-2.0 units (as defined by the supplier) of enzyme was added per µg of DNA per h of digestion.

Construction of Recombinant Plasmids and Phages—All plasmids and phages described here contain inserts derived from pGP2 whose construction (12) and nucleotide sequence (5) have been published. Refer also to Fig. 1. The gene for S20 is contained within a 550-bp HindII-HindIII fragment inserted into the gene conferring resistance to ampicillin in the vector pBR322 (13). The plasmid pGP3 was derived from pGP2 by cleaving the latter with HindIII and religating the larger fragment. This eliminates a 60-bp inverted repeat centered about the EcoRI site of the vector in pGP2. Additional plasmids whose structure is illustrated in Fig. 1 were constructed from pGP2 by attaching synthetic oligonucleotide linkers at specific restriction sites. We followed the procedure of Goodman and MacDonald (14) for repairing staggered ends (e.g. HindII or TnaI) for phosphorylating linkers and for ligating the linkers to DNA. In all cases, we started with approximately 3 pmol of target DNA and 100-150 pmol of linker monomers. We used GGAATTCC (Collaborative Research, Inc.) and GCAAGCTTGC (BRL, Inc.) as EcoRI and HindIII linkers, respectively. Following the ligation of the linkers to the target, the DNA was digested with the appropriate enzyme(s). The fragment of interest was purified by electrophoresis on polyacrylamide gels. It was subsequently eluted, concentrated, and ligated to the appropriate vector in a reaction containing 60 mM Tris-HCl, pH 7.5, 6 mM MgCl2, 10 mM dithiothreitol, 1 mM ATP, 0.025% gelatin in a volume of 0.01 ml at 13-15 °C for 5-16 h. The vector, previously cut with the appropriate restriction enzyme(s) and subsequently digested with calf alkaline intestinal phosphatase in the case of plasmids, was present at 50 ng/reaction, typically 0.014-0.018 pmol. DNA fragments to be cloned were present at an estimated 3-10-fold molar excess. The ligation was initiated with DNA ligase (0.3-0.5 unit from Boehringer

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†The abbreviations used are: bp, base pairs; PEI, polyethyleneimine.
Mannheim) and stopped with 0.015 M of 16.6 mM EDTA, pH 8. A portion of this mixture was used to transform the appropriate bacterial host previously rendered competent by treatment with CaCl2 (16). All candidate bacterial clones were purified by restreaking for single colonies and retesting the antibiotic resistance. Candidate recombinant plasmid phage were screened by electrophoretic mobility and plaque-purified. The latter procedure was especially important to avoid overgrowth of the recombinant by contaminating nonrecombinant phage in the original plaque.

The structures of the plasmids illustrated in Fig. 1 were confirmed by mapping with restriction enzymes. The position of the EcoRI linkers in pGM24 was confirmed by DNA sequencing (15) across the sites shown, followed by recloning of the truncated fragments as described under "Experimental Procedures" and "Materials and Methods." Further details are provided under "Experimental Procedures."
and Genetics, University of Guelph) for this suggestion.

Terminal nucleoside tetraphosphates were isolated from "run-off" transcripts eluted from a preparative polyacrylamide gel essentially as described by de Boer and Nomura (21). Labeled RNA was digested with a mixture of T1, T2, and pancreatic ribonucleases. The digest was applied to a PEI-cellulose thin layer plate (Machery-Nagel) and developed with 2.5 M NH₄-formate adjusted to pH 3.8 with formic acid. Pieces of PEI-cellulose corresponding to putative nucleoside tetraphosphates were cut from the chromatogram using an autoradiogram as a guide. The nucleotides were eluted with 30% triethylamine bicarbonate in H₂O with recoveries of 50–60%. The eluate was diluted with H₂O and concentrated by several cycles of lyophilization. Residues were dissolved in 18 μl of 50 mM NH₄-acetate, 10 mM Mg-acetate, 0.1 mM EDTA, 1 mM ATP, 1 mM GTP, pH 6.0. A portion was digested with 2 μg of P1 nuclease (P-L Biochemicals) for 75 min at 37 °C. Digested and undigested samples were analyzed by chromatography on PEI-cellulose using 2.5 M NH₄-formate adjusted to pH 3.8 as solvent. Nonradioactive markers were visualized by UV illumination.

DNA-directed Protein Synthesis—We employed Zubay's procedure (22) for coupled transcription and translation with slight modifications described previously (9, 23). The major departure from these conditions in the work described here was to limit the reactions with respect to DNA by using 1.0 μg/ml of plasmid DNA in reactions of 25 μl. This corresponds to approximately 0.3 nM DNA. For comparison, in Zubay's procedure as published, plasmid DNA is present at 50 μg/ml or 1.5 nM. Incubations were for 20 min at the indicated temperature and were stopped and desalted as described (23) except for the omission of DNase and RNase. Labeled proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (24). Gels were treated for fluorography, dried, and exposed. The developed x-ray film was used as a guide for excising pieces of acrylamide containing S20 from dried gels. These gel slices were rehydrated, washed successively with dimethyl sulfoxide, methanol, 50% methanol in water, and water to remove 2,5-diphenyloxazole. They were digested with Protosol (New England Nuclear) prior to liquid chromatography on PEI-cellulose using 2.5 M NH₄-formate adjusted to pH 3.8 as solvent. Nonradioactive markers were visualized by UV illumination.

RESULTS

In Vitro Transcription of the Gene for S20—The sequence of the gene for S20 and the location of RNA polymerase binding sites in "footprinting" experiments (5) suggest that transcription of the gene for S20 could be initiated at or near either residues 141 or 231 (using the numbering system in the published sequence). Refer also to Fig. 1. This prediction has been tested by transcribing, in vitro, DNA templates containing both RNA polymerase binding sites and a portion of the structural gene for S20 ending at the TaqI site at residue 388. Such templates should yield transcripts of approximately 250 or 160 residues if transcription initiates at site 1 or site 2, respectively. The data in Fig. 2 show that this expectation is the case using as template pGM32 cleaved with HindIII (see Fig. 1 and "Experimental Procedures"). At KCl concentrations below 160 mM, three distinct "run-off" transcripts are generated. Band 1 migrates with a mobility equivalent to a length of 250 residues, consistent with its initiation at site 1 in the gene for S20. Band 2 corresponds to an RNA of 160 residues, consistent with initiation in site 2. Band 3, corresponding to an RNA of 100 residues, is encoded by uncleaved pGM32 or by pBR322 alone (data not shown). These observations, as well as its 5' terminal sequence (data not shown), identify it as RNA I of Itoh and Tomizawa (25), an RNA involved in regulation of plasmid copy number.

The relative yields of Bands 1 and 2 change markedly as a function of KCl concentration present during transcription. The optimal KCl concentration for total incorporation and for the synthesis of Band 1 occurs at 120 mM (Fig. 2, lane c). In contrast, maximal yields of Band 2 occur at 40–80 mM KCl (Fig. 2, lanes a and b). At 80 mM KCl, the molar ratio of Band 2 to Band 1 is 2.0–2.3 (range in four experiments) whereas at 120 mM it falls to 0.8. It is difficult to extrapolate from these observations based on a linear template in vitro to the situation in vivo. Nonetheless, it is clear that both potential S20 promoters can direct the synthesis of RNA in vitro.

The Sequence of RNAs Synthesized in Vitro—We have taken two approaches to the identification of the starting points for initiation of transcription in sites 1 and 2. In the first, we found that both Bands 1 and 2 could be labeled in vitro with [γ-32P]GTP although the yield of Band 2 was low (data not shown). The RNA in these bands was eluted and then digested partially with one of the four ribonucleases described under "Experimental Procedures." Autoradiograms of the corresponding sequencing gels are illustrated in Fig. 3. The results show that the RNA in Band 1 is initiated with GTP at residue 141 in the sequence of S20. This agrees with a previous prediction (5) based on footprinting experiments. The RNA in Band 2 which can be labeled with [γ-32P]GTP is initiated in site 2 at residue 231 of the S20 sequence. In both cases, the sequences of the RNA transcripts agree, to the extent to which the sequence can be read, with the published DNA sequence.

Our second approach to determining the point of initiation of transcription of the gene for S20 was prompted by the occurrence of cytidine nucleotides at the 5' termini of other transcripts (6, 21). We transcribed HindIII-cleaved pGM32 in the presence of one of three radioactive triphosphates (refer to the legend of Fig. 4) and subsequently isolated the labeled RNA in Bands 1, 2, and 3. Digestion of each labeled RNA with ribonucleases A, T1, and T2 released mononucleotides and the 5' terminal nucleoside 5'-triphosphate, 3'-monophosphate as shown in Fig. 4. The structure of the putative nucleoside tetraphosphates was confirmed by converting them

FIG. 2. Transcription in vitro of HindIII-cleaved pGM32. Transcription was performed as described under "Experimental Procedures" at the following concentrations of KCl: (a) 40 mM, (b) 80 mM, (c) 120 mM, (d) 160 mM, (e) 200 mM. The template was HindIII-cleaved pGM32 (refer to Fig. 1) while [γ-32P]GTP was used to label the RNA. The numbers in the left-hand margin give the sizes in residues of markers, namely end-labeled HinfI fragments of pBR322. The numbers in the right-hand margin identify Bands 1, 2, and 3 (see the text), whose sizes are approximately 250, 160, and 100 residues, respectively.
levels of radioactivity in spot 2 prevented us from measuring the $^{32}$P released during P1 digestion. We therefore cannot exclude the possibility of a low level of initiation at residue 143.

A faint spot in Fig. 4c was identified as pppAp. At present, we believe that this represents a contaminant in Band 1.

**Fig. 4.** Analysis of 5′ terminal nucleoside tetraphosphates from in vitro transcripts. HindIII-cleaved pGM32 was transcribed in vitro at 120 mM KCl in the presence of one labeled nucleoside triphosphate (see below) as described under “Experimental Procedures.” Bands 1 and 2 were eluted from a gel (cf. Fig. 2e) and digested with a mixture of T1, T2, and pancreatic ribonucleases. Samples were applied to PEI-cellulose thin layer plates and developed with 2.8 M NH$_4$-formate, pH 3.8 (21). Lanes a, b, and c illustrate products obtained from Band 1; lanes d, e, and f those from Band 2. The transcripts were labeled as follows: lanes a and d, [γ-$^{32}$P]GTP; lanes b and e, [α-$^{32}$P]GTP; lanes c and f [α-$^{32}$P]UTP. Each labeled nucleotide was present at essentially the same specific activity. O represents the origin, ms1, the position of marker ppGpp, and Np, the position of nucleoside 3′-monophosphates.

**Fig. 5.** Digestion of nucleoside tetraphosphates with nuclease P1. Putative nucleoside tetraphosphates from the separation illustrated in Fig. 4 were eluted and concentrated (see “Experimental Procedures”). Portions of each were digested with nuclease P1 (lanes a, c, e, g, i, k) while the remainder (½ the total) (lanes b, d, f, h, j, l) served as an undigested control. Samples were subjected to chromatography on PEI-cellulose in 2.5 M NH$_4$-formate, pH 3.8. The central margin illustrates the position of marker nucleoside triphosphates. o represents the origin, and ms1, the position of ppGpp. Lanes a and b, Band 1 spot 1; lanes c and d, Band 1 spot 2; lanes e and f, Band 1 spot 3; lanes g and h, Band 2 spot 4; lanes i and j, Band 2 spot 5; lanes k and l, Band 2 spot 6 (refer also to Fig. 4).
While Band 1 could be labeled weakly with [\(\gamma^3P\)]ATP, the level of incorporation was too low to permit the sequencing of the labeled component. Analysis of Band 2 yielded the unanticipated result that initiation of transcription occurred predominantly with CTP, regardless of KCl concentration during transcription. Digestion of [\(\alpha^32P\)]CTP-labeled Band 2 with T2 ribonuclease yielded both pppCp and pppGp (Fig. 4d) in the relative proportions of 2:1, respectively, at 130 mM KCl (average of two separate experiments). At the optimal KCl concentration for synthesis of Band 2 (80 mM), this ratio rose to 4:1 in favor of initiation with CTP (data not shown).

Several pieces of evidence suggest that transcription can initiate with CTP at residues 232 or 233. First, labeling of Band 2 with [\(\alpha^32P\)]UTP resulted in the transfer of label to the 3' phosphate of pppCp (spot 6 in Fig. 4f; see also Fig. 5, k and l). This is consistent with initiation at residue 233. The second line of evidence is based on the recoveries of P, from pppCp. When Band 2 is labeled with [\(\alpha^32P\)]CTP, an initiation at residue 232 will yield a transcript pppCpCpUp... whose terminal tetraphosphate will be labeled in both the 5' a and the 3' phosphates, pppCp from a transcript initiated at residue 233, in contrast, will be labeled only on the 5' a phosphate. Digestion of pppCp with P1 will release the 3' phosphate (cf. Fig. 5, g and h). In an experiment similar to that illustrated in Fig. 5g (but from Band 2 labeled with [\(\alpha^32P\)]CTP at 80 mM KCl), the yield of \(\gamma^3P\) was 228 cpm, and of CTP, 344 cpm. Thus, 69% of the transcripts initiated with CTP in Band 2 did so at residue 232, the balance at residue 233.

The results of these experiments confirm clearly that the gene for S20 contains two promoters active in vitro. At low ionic strengths, site 2 is at least twice as active as site 1. While both promoters can initiate with CTP, it is clear that CTP is the preferred nucleotide for site 2, reminiscent of the tandem promoters in rRNA operons (21).

**Promoter Activity of Sites 1 and 2 Assayed by Operon Fusion**—In order to assess the activities of the promoters in sites 1 and 2 separately, we have attached synthetic oligonucleotide linkers to appropriate restriction sites in the gene for S20 and have recloned the corresponding fragments between the EcoRI and HindIII sites of pBR322. In such plasmids, part of the promoter for the gene(s) conferring resistance to tetracycline is deleted. Expression of tetracycline resistance should, therefore, reflect the promoter activity of the EcoRI-HindIII fragment spanning residues 232–551 from pGM24 and recloned into pBR322 (26), to yield pGM31 (Fig. 1) effectively placing the expression of S20 as well as resistance to tetracycline under control of the lac operator-promoter system. Cells harboring this plasmid, strain G2M65, are resistant to about 10 mM/ml of tetracycline in the presence of isopropylthio-\(\beta\)-galactoside. Assays of \(\beta\)-galactosidase showed that under these conditions the chromosomal lac operon is fully induced. It is clear from these experiments that both sites 1 and 2 contain promoters and that these promoters, singly or together, are substantially more active than the wild type lac promoter.

The limitations of this method of assaying promoter efficiency are several. First, we have not corrected our results for copy number. Perhaps, more importantly, we cannot evaluate the influence of factors other than promoter strength which could affect the observed level of resistance to tetracycline (see An and Frissen (27, 28) for further discussions of this point). We point out, nonetheless, that pGM28 which contains two copies of the 160-bp fragment containing site 1 oriented correctly with respect to the structural gene confers resistance to 40 \(\mu\)g/ml of tetracycline, exactly double its single copy counterpart, pGM26. Plasmid pGM29 which contains three copies of site 1 confers resistance to 50 \(\mu\)g/ml of tetracycline. These data suggest that our assay can successfully respond with near linearity to different promoters. A final complication in this approach generally is that the use of linkers effectively alters the wild type sequence. This, together with the need to employ convenient restriction sites for these constructions, means that we may have altered or deleted sites normally important in the expression of S20. For example, the fragment containing site 1 has been constructed in a way which eliminates a short inverted repeat sequence centered on residues 168–169 in the sequence of S20. The removal of this sequence in pGM26 could lead to an overestimate of the activity of site 1 if pausing at this inverted repeat were significant in the normal expression of S20.

### Table 1

**Resistance to tetracycline conferred by different plasmids**

Fresh, saturated cultures of the strains tested below were grown in supplemented M9 medium containing, where appropriate, ampicillin at 30 \(\mu\)g/ml. A loopful of each was streaked across an L-broth agar plate containing tetracycline at final concentrations of 1–100 \(\mu\)g/ml. Each strain was tested at a given concentration of tetracycline on the same plate. The plates were incubated at 37 °C for 18–20 h and scored for growth. The end point for growth was defined as the highest concentration of tetracycline where there was no visible inhibition of the size of the streak of bacteria. Similar results were obtained with growth at 29 °C or 42 °C, or with scoring growth at different times after streaking. This test was repeated three times with similar results.

| Strain* | Plasmid | Level of resistanceb |
|---------|---------|-----------------------|
| GM357   | pGM23   | 100                   |
| GM259   | pGM25   | 50                    |
| GM260   | pGM26   | 5                     |
| GM261   | pGM27   | 1                     |
| GM258   | pGM24   | 1                     |
| GM258   | pBR322  | 70                    |
| GM265   | pGM31   | 10                    |
| MM294   | pGM32   | 1                     |

*All strains are derivatives of MM294, differing only in the plasmids, the structures of which are summarized in Fig. 1.

bLevel of resistance is the concentration of tetracycline in micrograms/ml at the growth end point as defined above.
Efficiency of the Promoters in Sites 1 and 2 in Directing Coupled Transcription and Translation—We have measured the efficiencies of the promoters in sites 1 and 2 in directing the synthesis of S20 by using the plasmids described above as templates for DNA-directed protein synthesis. In order to maintain dependence on DNA, it was necessary to use lower concentrations of template than is commonly the case in such experiments, namely 1 μg/ml (approximately 0.5 mM). Table II summarizes the rates of synthesis of S20 dependent on different templates measured at either 30 °C or 37 °C. At both temperatures as well as at intermediate temperatures (data not shown), the promoters in pGM23 appear to function additively (and thus independently) since the rate of synthesis of S20 dependent on pGM23 is essentially the sum of the rates dependent on pGM25 and pGM26. In this assay, the promoter in site 2 (i.e. in pGM25) is from 5- to 9-fold more active than the promoter in site 1. A second point which emerges from this experiment is that when the promoter in site 1 is arranged in tandem repeated copies, as in pGM28 and pGM29, the resultant rates of synthesis of S20 are in proportion to the number of promoters (compare lines 3–5 in Table II). Apart from confirming the linearity of the rate of synthesis of S20 with promoter availability in this assay, the results of this experiment further demonstrate that tandem promoters can function independently and essentially additively.

We have also measured the rate of synthesis of S20 dependent on the lac promoter in plasmid pGM31. Even in the presence of inducer and cyclic AMP, the rate of synthesis of S20 (Table II, line 6) was less than half the rate dependent on pGM26 and only 4% of the rate dependent on pGM23. In the absence of inducer, the rate of synthesis of S20 was too low to be measured reliably and was indistinguishable from the rate dependent on pGM24 which lacks both S20 promoters.

Wirth and Böck (29) and Wirth et al. (30) have reported that the addition of 18 S rRNA to an in vitro reaction similar to ours will stimulate the synthesis of S20, presumably by relieving translational repression. We have found that 100 pmol of pure S20 are required to elicit a 40% inhibition of the rate of synthesis of S20 at 30 °C at 1 μg/ml of pGM23 (data not shown). This inhibition is less pronounced at 37 °C. In contrast, the maximum accumulation of S20 in these experiments was 1.7 pmol at 37 °C. We believe, therefore, that the data in Table II do not underestimate promoter activities as a consequence of translational repression.

Promoter Selection in Vivo Assayed by Annealing—We have asked whether the preference for site 2 implied by the preceding experiments is actually the case in haploid strains in vivo. To distinguish between transcripts initiated at site 1 and those at site 2 we have used M13GM3 as probe (refer to Fig. 1). This will anneal only to transcripts initiated at site 1. M13GM2, in contrast, will detect both classes of transcript. Under conditions of DNA excess (12, 31), 0.104% of pulse-labeled RNA from a haploid strain doubling in 39 min annealed to M13GM2 DNA while 0.0075% annealed to M13GM3 DNA (the average of duplicate determinations in two separate experiments). Making the assumption that annealing efficiencies are comparable for the two probes and correcting for the sizes of sequences which can be detected by each, we calculate that under these conditions of growth 25% of all S20 transcripts initiate at site 1. Thus, it would appear that under these conditions of growth, site 2 is 3 times more active in vivo than site 1, in reasonable agreement with our other measurements.

### DISCUSSION

Structure and Relative Activities of the Tandem Promoters for S20—We have previously proposed that the gene for S20 includes two promoters. This was based on the structural homologies of two regions of the DNA sequence (site 1 and site 2) with known promoters and on the protection of these sites by RNA polymerase in “footprinting” experiments (5). The present work demonstrates that the DNA sequences in sites 1 and 2 can function as promoters in the sense that each is competent to initiate transcription of DNA into a defined RNA molecule in an in vitro reaction whose exclusive macromolecular components are DNA and RNA polymerase. The assays which we have employed to measure the activities of the S20 promoters in vitro or in vivo, yield qualitatively similar results, specifically, that site 2 contains the more active promoter. Despite its lower activity, the promoter in site 1 still makes a substantial contribution to the overall rate of synthesis of S20 mRNA, from 10–25% of the total. In contrast, in the rrrE gene, the first promoter may account for up to 90% of the total transcriptional initiation (21).

While there are precedents for tandem promoters in E. coli (for example, in the rRNA genes (21, 32–34), for CTP starts (6, 21, 32–35) and for heterogeneity in the initiating nucleotide (36), finding all three together in an apparently simple gene is remarkable. The promoters in both sites 1 and 2 exhibit heterogeneity in the initiating nucleotide. In site 1, initiation occurs primarily (80–85%) with GTP at residue 141 and to a limited extent with CTP at residue 142. In site 2 which contains the more active promoter, the degree of heterogeneity in the initiating nucleotide is even more striking. We estimate that, at 80 mM KCl, 55% of all transcripts from site 2 initiate with CTP at residue 232, 25% (or fewer) with CTP at residue 233, and up to 20% with GTP at residue 231. What features of the interaction between RNA polymerase and DNA contribute to the observed heterogeneity are unknown. It is surprising, however, that the DNA sequences spanning the preferred sites of initiation in both site 1 (residues 135–143) and site 2 (residues 225–233) (Ref. 5; see also Fig. 6) are virtually identical. Some subtle feature of the DNA sequence in the promoter, likely in the “-35” and “-10” regions, must

| Template | Promoter(s) | Additions | Rate of synthesis of S20* |
|----------|-------------|-----------|--------------------------|
| pGM23    | Sites 1 + 2 |           | 360 (11.6) 1215 (7.0)    |
| pGM25    | Site 2     |           | 263 (8.5) 909 (6.3)      |
| pGM26    | Site 1     |           | 31 (1.0) 177 (1.0)       |
| pGM28    | 2 × Site 1 |           | 65 (2.0) 366 (2.1)       |
| pGM29    | 3 × Site 1 |           | 156 (5.0) 482 (2.8)      |
| pGM31    | lac        | 1 mM IPTG b| 1 mM cAMP              |

*The rate of synthesis of S20 is defined as the picomoles of [35S] methionine incorporated into S20 per pmol of template during a 20 min incubation at the stated temperature. The specific activity of methionine was 2700 cpm/pmol. All the data represent the average of two independent experiments except for pGM26 for which four experiments were averaged. The numbers in parentheses give the rate of synthesis of S20 dependent on a given template relative to the rate dependent on pGM26 at a given temperature.

bIPTG, isopropylthio-D-galactoside.
Fig. 6. Structure of the tandem promoters for S20. The coding strands of DNA (whose sequence is taken from Ref. 5) in the promoters of sites 1 and 2 are aligned with respect to the initiating nucleotide determined in this work. Those nucleotides representing the best fit with a consensus sequence at -10 (39) are underlined (solid line). Three possible -35 sequences in site 1 are underlined with dotted lines. We point out that while TTCACA at residues 111-116 provides the best fit to a -35 consensus sequence, it may be too close to the -10 sequence to function as such (Refs. 6-8; Footnote 2). The heavy arrows above the coding strand indicate the major sites of transcriptional initiation; thin arrows denote minor starts (see the text).

Determine the degree of heterogeneity in transcriptional initiation. The significance, if any, of such microheterogeneities remains to be determined.

The initiation of transcription of the gene for S20 occurs with CTP in 55-60% of all transcripts initiated in vitro (at 80 mM KCl). Transcription of another ribosomal protein operon (rpL, rpS, rpoA, rpoB, rpoC) also initiates with CTP (35). Since the latter operon and the gene for S20 encode relatively abundant gene products, it is clear that initiation of transcription with CTP is not inherently disadvantageous. There is no reason to believe at present that initiation with CTP is of regulatory significance.

Independent Functioning of Sites 1 and 2—Several investigations have shown that tandem promoters can interact negatively. In one example, the P2 promoter of the rnr gene can promote the stalling of an elongating RNA polymerase which initiated transcription at the first promoter (37). In another, one (or other) of the gal promoters can be occluded by elongating RNA polymerases which have initiated at a highly efficient “upstream” promoter, in this case, AP1 (38). Neither of these situations occurs to seem in the gene for S20. First, “footprinting” experiments show that both promoters can be occupied simultaneously (Fig. 4a in Ref. 5) in the absence of elongation. Secondly, the results obtained in this work are best explained by postulating the independent additive functioning of the two promoters. Why the gene for S20 possesses and uses two independent promoters is a question which must remain open to speculation.

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