Species Specificity of Promoter Recognition by RNA Polymerase and Its Transfer by the Sigma Factor

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RNA polymerase holoenzyme from Micrococcus luteus synthesizes in vitro a run-off transcript of 85 nucleotides from a DNA fragment containing part of gene E of bacteriophage φX174. This RNA starts with GTP as the 5' terminus 18 nucleotides downstream from the start of gene E on the viral (+) strand. Transcription does not occur when the fragment is cleaved 36 nucleotides upstream of the initiation site. No transcript is obtained with RNA polymerase core or holoenzyme from Escherichia coli. Other DNA fragments containing the three major E. coli promoters of φX174 are transcribed by both enzymes although much less efficiently by M. luteus RNA polymerase. When subunit sigma in E. coli RNA polymerase is replaced by sigma from M. luteus the resulting hybrid enzyme actively transcribes the DNA fragment containing the inner region of gene D with formation of the same run-off transcript which is obtained with M. luteus holoenzyme. In the presence of sigma from E. coli this RNA is not synthesized. The hybrid enzyme also transcribes a DNA fragment containing the gene A promoter of φX174 with even higher efficiency than RNA polymerase holoenzyme from E. coli.

RNA polymerase holoenzymes from bacteria recognize specific sites on double-stranded DNA from which they start to transcribe the template [1]. Upon comparison of the transcription products obtained with T7 bacteriophage DNA and RNA polymerases originating from a wide range of microorganisms it was found that all these enzymes recognize the strong promoters on the left end of the phage genome [2]. This implies that the recognition of a promoter sequence is independent of the eubacterial species.

To check the general validity of this conclusion we have compared RNA polymerase from Micrococcus luteus [3] with that from Escherichia coli with regard to the specificity of transcription initiation on various double-stranded DNA fragments derived from the DNA of bacteriophage φX174 whose sequence is known [4,5]. We found that RNA polymerase from M. luteus is responsible for high-rate synthesis of a transcript on a DNA fragment which is not transcribed by either E. coli holoenzyme or core enzyme. The same transcript is produced upon addition of subunit sigma from M. luteus RNA polymerase to core enzyme from E. coli.

**MATERIALS AND METHODS**

**Enzymes and Nucleic Acids**

RNA polymerase core and holoenzyme and subunit sigma from Escherichia coli and RNA polymerase holoenzyme and subunit sigma from Micrococcus luteus were prepared according to Burgess and Jendrisak [6] and Lill et al. [3] respectively. The fragments of φX174 RF I DNA (New England Biolabs GmbH, Schwalbach) were obtained by digestion with commercially available restriction endonucleases and isolated according to Lederboer et al. [7].

**Transcripts**

50 mM Tris/HCl pH 7.9, 0.1 mM dithioerythritol, 10 mM MgCl2, 0.2 M KCl (if not otherwise indicated), 0.6 mM each of ATP, GTP and CTP, 0.16 mM [α-32P]UTP (400 Ci/mmol; Amersham-Buchler, Braunschweig), 13 nM DNA restriction fragment (as indicated) and 52 nM RNA polymerase holoenzyme (as indicated or 52 nM RNA polymerase core enzyme from E. coli + 0.26 µM sigma from M. luteus in experiments with enzyme hybrid) were incubated for 30 min at 37°C. The concentration of glycerol in the incubation mixture was less than 1% [8]. The transcription was stopped by addition of an equal volume of 0.2 mg/ml brewer's yeast tRNA (Boehringer, Mannheim) containing 50 mM EDTA at 0°C.

When transcription was primed with dinucleotides [9] the concentration of ATP, CTP and [α-32P]UTP (total amount of radioactivity added remained unchanged) was lowered to 25 µM, that of GTP to 10 µM, 0.2 mM dinucleoside phosphate (Sigma Chemie, München) was added as indicated.

For the preparation of RNA 5'-labelled with [γ-32P]GTP, the incubation mixture (total volume 0.1 ml) contained 0.1 mM [γ-32P]GTP (25 Ci/mmol; Amersham-Buchler, Braunschweig) instead of 0.6 mM GTP. [α-32P]UTP was replaced by 0.4 mM UTP. The concentration of enzyme and DNA fragment was increased 2.5-fold, the time of incubation to 90 min. Addition of tRNA/EDTA at the end of the incubation was omitted.

The reaction products were precipitated with ethanol and dissolved by heating to 90°C for 2 min in 36 mM Tris-phosphate pH 7.7 containing 10 mM EDTA, 8 M urea, 0.5% sodium dodecylsulfate and 10% glycerol [10].
Analysis of the labelled products was carried out by electrophoresis in slab gels containing 8% acrylamide, 7 M urea and 0.1 M sodium dodecylsulfate in 36 mM Tris/phosphate pH 7.7. Radioactivity was detected by autoradiography and recorded densitometrically. To determine the size of the transcripts the electrophoretic mobility was compared with that of standards (tRNAval and tRNAfyr and 5-S rRNA from E. coli, obtained from Boehringer, Mannheim).

**Sequence Analysis**

To determine the 5'-proximal nucleotide sequence 5'-[γ-32P]GTP-labelled RNA (2 × 10^4 counts × min⁻¹) was eluted from the polyacrylamide gel similarly as described in [11], further purified by electrophoresis in gels containing 20% acrylamide and 7 M urea similar to [12], eluted as before with addition of 30 μg tRNA (Boehringer, Mannheim) and precipitated with ethanol. After degradation by limited acid hydrolysis [13], the resulting oligonucleotides were fractionated by electrophoresis on cellulose acetate (Schleicher & Schüll, Dassel) and radioactivity was detected by autoradiography.

**Ribonuclease T1 Digest**

[γ-32P]GTP-labelled transcript (2 × 10^4 counts × min⁻¹) was partially digested with ribonuclease T1 (Calbiochem GmbH, Giessen) similarly as described by Krupp and Gross [13]. The resulting oligonucleotides were separated by electrophoresis in 0.4-mm-thick slab gels containing 20% acrylamide and 7 M urea similar to [12]. Radioactivity was detected by autoradiography.

**RESULTS**

**Initiation of Transcription at a Micrococcus luteus Specific Site**

The fragment HaelIII-7 of the φX174 genome comprising the base pairs 436 – 669 is located within genes D and E and does not contain a known promoter for *Escherichia coli* RNA polymerase [4] (Fig. 1). When this fragment is incubated with a fourfold excess of RNA polymerase holoenzyme from *M. luteus* in the presence of [γ-32P]UTP, ATP, CTP and GTP, a single prominent transcript is generated, as is revealed by electrophoresis in gels containing 8% acrylamide and 7 M urea similar to [12]. Radioactivity was detected by autoradiography.

The fragment *HaeIII*-7 of φX174 by RNA polymerase holoenzyme from *E. coli* or *M. luteus* respectively at different concentrations of KCl. Transcription with RNA polymerase holoenzyme from *E. coli* (a, c, e, g) or *M. luteus* (b, d, f, h) in the presence of 50 mM (a, b), 100 mM (c, d), 150 mM (e, f) and 200 mM (g, h) KCl.

![Fig. 1. Restriction pattern of gene D and E of φX174 (according to [4]).](image)

**Fig. 2. Transcription of fragment *HaeIII*-7 of φX174 by RNA polymerase holoenzyme from *E. coli* or *M. luteus* respectively at different concentrations of KCl.**
mobility of these nucleotides is due to the presence of a 5'-triphosphate group. To confirm this assignment the left neighbour of the 5'-terminal guanine of the transcript was determined by an independent method. Downey et al. [17] and Minkley and Pribnow [9] have shown that in vitro and at low concentration of the starting purine ribonucleoside triphosphate, specific RNA synthesis can be primed by addition of the template-directed dinucleotide at the initiation site. The highest effect is observed with a dinucleotide containing the 5'-terminal nucleotide of the unprimed transcript in the 3' position [9]. Therefore the priming efficiency of UpG, ApG, GpG and CpG was compared. In these experiments the fragment Taq-5 was used as template. A specific RNA which has the same length as the specific transcript of the unprimed reaction is only obtained with UpG as primer (Fig. 4). These data suggest the sequence dTpdG at the initiation site of the non-coding DNA strand which is colinear with the RNA. Combining the results of Fig. 3 and 4 and comparison with the known sequence of the fragments HaeIII-7 and Taq-5 [4] shows unequivocally that the sequence of the RNA obtained in the unprimed reaction is colinear with the viral (+)-strand and starts at position 585 as 5'-terminal nucleotide.

585 595

located 18 nucleotides downstream from the beginning of the coding sequence of gene E [4].

The elucidation of this RNA initiation site together with the total chain length of the transcripts (90, 140 and 230 nucleotides obtained with the fragments HaeIII-7, Taq-5 and MboII-5 respectively) suggests that all these RNAs are run-off transcripts where termination of transcription is caused by the end of the DNA fragments at base pair 669, 721 and 816 respectively.

The formation of a species-specific transcript with fragment MboII-7 [4] suggests that sequences upstream of nucleotide 493 (Fig. 1) are not required for the M. luteus specific initiation of transcription at base pair 585. To learn how much of the remaining upstream sequences is required, the isolated fragments Taq-5 or HaeIII-7 were digested with the restriction endonuclease Fnu4HI which cleaves these fragments 36 nucleotides upstream and 40 nucleotides downstream of the initiation site (Fig. 1). No specific transcript is made on these cleaved fragments with M. luteus RNA polymerase under synthesizing conditions. This indicates that sequences more than 36 base pairs upstream of the initiation site are essential for the M. luteus specific initiation of transcription.

Initiation at E. coli Specific Sites

The observation of a specific initiation of transcription by M. luteus RNA polymerase at base pair 585 [4] of the ϕX
Fig. 5. Transcription of the restriction fragment HaeIII-3 containing the promoter for gene D with the RNA polymerase holoenzyme from M. luteus (right track) or E. coli (left track). The electrophoretic mobilities of marker RNAs are indicated.

Promoter Specificity of a Hybrid Enzyme

In Bacillus subtilis various proteins with sigma-like activity exist [18–21]. During transcription with B. subtilis RNA polymerase in vitro the specificity of promoter recognition strongly depends on the kind of B. subtilis sigma present in the incubation mixture and can be altered by substitution with different sigmas. Therefore the question arises whether specificity in the initiation of transcription is transferable from one bacterial species to another by exchange of sigma subunits. If so, isolated subunit sigma of the M. luteus enzyme should be able to confer the specificity to recognize the initiation site of transcription at base pair 585 of the \( \phi X \) genome to E. coli RNA polymerase core enzyme. It has been shown earlier that M. luteus sigma can form an active hybrid holoenzyme with E. coli core polymerase [22]. When the fragment HaeIII-7 is incubated with E. coli core enzyme and micrococcal sigma in the presence of substrate a specific transcript is formed which migrates with the same electrophoretic mobility as that seen with the M. luteus enzyme. These results show that the very intense M. luteus specific transcript obtained with fragment HaeIII-7 (Fig. 2) is not due to a higher number of active enzyme molecules in the preparation of the M. luteus enzyme used compared with that in the preparation of the E. coli enzyme but rather the result of a species specificity in the initiation of transcription.
phoretic mobility as the RNA synthesized by M. luteus holo-
enzyme (either native or reconstituted). E. coli core enzyme
alone is as inactive under these conditions as the E. coli holo-
enzyme (Fig. 6). M. luteus sigma alone or the hybrid from
M. luteus core enzyme and E. coli sigma (data not shown).
The total amount of radioactivity (1.5 x 10^4 counts x min^-1)
incorporated into the specific transcript by the hybrid enzyme
is about half of that obtained with the micrococcal holo-
enzyme (3.0 x 10^4 counts x min^-1). This species-specific effect
of M. luteus sigma is suppressed when isolated sigma from
E. coli is simultaneously present in the incubation mixture
in threefold excess (Fig. 6). This observation supports the
notion that the two sigmas are competing for the same site
on the E. coli core polymerase. Radioactive labelling of the
specific transcript synthesized by the hybrid enzyme can be
achieved with [^32P]GTP (Fig. 7). This clearly shows that the 5' terminus of the transcript starts with GTP just as the
RNA made by the M. luteus holoenzyme. Furthermore,
specific transcription of the fragment TaqI-5 by the hybrid
enzyme is stimulated by the dinucleotide UpG just as de-
scribed for the RNA synthesized by M. luteus holoenzyme
(Fig. 4). Additional evidence that the transcript made by the
hybrid enzyme is identical with that obtained by M. luteus
holoenzyme is provided by limited digestion of the RNA
with the guanine-specific ribonuclease T1. When the tran-
scripts labelled with [^32P]GTP are digested and the reaction
products are separated according to size by electrophoresis
both ladders of radioactive spots exhibit the same pattern
(Fig. 7). These results show that the species specificity in the
initiation of transcription of M. luteus RNA polymerase can
be transferred to E. coli core enzyme by M. luteus sigma.

Such an effect may be achieved by M. luteus sigma in
different ways. One possibility is a general enhancing activity
on the initiation of transcription. In this case M. luteus sigma
should not only increase the initiation of transcription at
M. luteus specific promoters but also at E. coli promoters by
E. coli RNA polymerase core enzyme. To answer this question
the restriction fragment HindII-4 containing the
promoter of gene A [4, 5] was incubated under synthesizing
conditions with E. coli core enzyme and M. luteus sigma,
E. coli or M. luteus RNA polymerase holoenzyme respectively
(Fig. 8). Both parental enzymes (either native or reconstituted
from core enzyme and sigma) recognize the promoter of
gene A resulting in the synthesis of the same run-off transcript
although distinctly less is synthesized by M. luteus holo-
enzyme as has been mentioned above. The hybrid enzyme,
however, is most active not only in synthesizing the gene A
transcript but also the minor RNAs made by either of the
parental enzymes (Fig. 8). Since neither E. coli core enzyme
nor M. luteus sigma are active in the transcription of the
HindII-4 fragment the high activity observed must be due
to the hybrid enzyme. These results show that the enhancing
effect of the micrococcal sigma on E. coli core enzyme be-
comes apparent not only with micrococcal promoters but also
with E. coli promoters.

DISCUSSION

Bacterial RNA polymerases efficiently recognize certain
nucleotide sequences as promoters provided these sequences
exhibit the essential features of a promoter sequence of the
organism from which the polymerase is derived [1]. The
source of the DNA with such sequences is irrelevant allowing, for example, the RNA polymerase from Escherichia coli to transcribe SV40 DNA [23]. It follows that any DNA can be used as a template to look for species specificity in promoter recognition by RNA polymerase. Indeed, our experiments show that φX174 DNA contains a sequence which functions as a promoter specific for Micrococcus luteus RNA polymerase, thereby leading to synthesis of a specific RNA. Although this RNA as well as the RNAs transcribed by E. coli RNA polymerase [24] are all initiated from the viral (+)-strand, the ‘micrococcal’ RNA is not visibly synthesized by the enzyme from E. coli. Obviously, M. luteus RNA polymerase shows a distinct species specificity in promoter recognition consistent with previous observations made with RNA polymerase from Caulobacter crescentus [25] or Bacillus subtilis [26]. Therefore the observation that the strong promoters on the left end of T7 bacteriophage DNA are recognized by numerous eubacterial RNA polymerases [2] cannot be generalized for all promoters.

The observation of a distinct species specificity in the initiation of transcription is important for the expression of cloned foreign genes. Nucleotide sequences on a cloned gene which do not function as promoters in the donor organism may act as promoters for the host RNA polymerase and thereby interfere with correct and efficient gene expression.

Comparing the large number of E. coli promoters [1] with each other it becomes clear that an unequivocal ‘promoter code’ similar to the genetic code for amino acids does not exist. In fact, rather divergent sequences can function as promoters. This should not be surprising since promoter activity is based on interactions between nucleic acid and protein and not on base pairing. Furthermore, the effective interaction of the polymerase with the template in promoter recognition extends over more than 40 base pairs upstream of the initiation site [27]. Interactions over such an extended region should allow for a certain variability in the sequences without loss of promoter function. Nevertheless, certain restrictions must exist, otherwise RNA synthesis would not be initiated exclusively by M. luteus RNA polymerase on the left end of gene E of φX174. This fact suggests searching for particular deviations of the M. luteus specific promoter sequence from the consensus sequence for E. coli RNA polymerase [1]. Recognition of M. luteus specific sequence can be prevented by cleaving the template 36 base pairs upstream of the initiation site at base pair 585 [4,5]. Obviously nucleotide sequences up to at least this position function in the recognition process, as is also the case for E. coli RNA polymerase [1].

Upon comparison of the sequences upstream of the initiation sites of the three main transcripts of φX174 DNA with the upstream sequence of the M. luteus specific initiation site, only the E. coli promoter of gene B [4] shows a distinct sequence homology, particularly in the region −8 to −21 (homologies are printed in bold-face type). In addition, the 3′-terminal is conversed.

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E. coli promoter of gene B

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M. luteus promoter within gene E

Less homology is found around nucleotide −35 although the frequently observed dinucleotide dTpG is also conserved. This consensus between the two promoters may explain why M. luteus RNA polymerase, albeit inefficiently, recognizes some E. coli promoters such as those prior to the genes A, B and D of φX174. Others, such as the promoter of the β-lactamase gene on pBR322, are almost not recognized (R. Brack and G. R. Hartmann, unpublished experiments). On the other hand, these homologies are not sufficient for the recognition of the M. luteus promoter by the E. coli enzyme. Possibly, the dG + dC content of the region around the Pribnow box and the recognition site is too high for the E. coli enzyme whose promoters tend to be (dA + dT)-rich in these regions. Furthermore, it should be noted that M. luteus DNA itself is particularly rich in dG + dC (72 mol% [26]).

When the sigma subunit of E. coli RNA polymerase is exchanged for sigma from M. luteus [22] the resulting chimera efficiently starts transcription at the M. luteus specific promoter within gene E of φX174. This observation clearly shows that specifies specificity in the initiation of transcription is very much a function of the subunit sigma of eubacterial RNA polymerases. Evidently sigma can transfer this specificity even from a gram-positive to a gram-negative bacterium. It points simultaneously to the general functional similarity of eubacterial RNA polymerases [29] as well as to their difference in promoter recognition.

How sigma modifies the interaction of the core enzyme with the promoter is not yet known although experiments with modified DNA [27] and crosslinking experiments [30] demonstrate a direct participation of sigma in the physical interaction of the enzyme with the promoter. Not only the equilibrium of the association of the enzyme with the promoter but also kinetic constants in the process of initiation may be altered [31] by the sigma used. Perhaps M. luteus sigma enhances the melting of the DNA in the enzyme-promoter complex believed to be involved in the initiation reaction [32]. Consistent with this hypothesis is the observation that transcript formation from E. coli promoters by E. coli RNA polymerase is also enhanced by the replacement of E. coli sigma by M. luteus sigma. Obviously, the function of sigma exceeds that concerned with the species specificity in promoter recognition.

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