LOCALISATION OF THE TRANSCRIPTION INITIATION SITE OF THE CHLORAMPHENICOL RESISTANCE GENE ON PLASMID pAC184

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1. Introduction

In *Escherichia coli*, R-factor carried resistance to chloramphenicol is exerted by acetylation of the antibiotic by the enzyme chloramphenicol acetyl transferase [1]. An unusual feature of the *cat* gene is its catabolite sensitivity, a feature more commonly associated with *lac, gal* and other sugar operons [2,3]. Catabolite sensitivity of the *cat* gene has been demonstrated both in vivo and in vitro [4]: however, since then there has been little biochemical or genetic analysis on such regulation of the *cat* operon.

Here we have studied transcription of the *cat* gene of plasmid pAC184 [5]. These studies were suggested by and subsequently compared to nucleotide sequence studies of an analogous *cat* gene as well as electron microscopic analysis of transcription of pAC184, both carried out by independent research [6] (D. Stüber and H. Bujard, personal communication). Our experiments have demonstrated that the predominant RNA species produced from a 1.5 kb *Hin*dIII/*Eco* RI restriction fragment of pAC184 is ~270 nucleotides long. When this fragment is transcribed in presence of cAMP and CRP, the rate of synthesis of the 270 nucleotide transcript is markedly enhanced, an effect even more noticeable when *Eco* RI-cut, linearised pAC184 is used as template.

The results of our transcription studies and a restriction analysis of the appropiate part of pAC184 allow us to locate the promoter of the *cat* operon to the right of the *Eco* RI site of this plasmid and to predict the transcription to proceed counterclockwise.

Abbreviations: cAMP, cyclic adenosine monophosphate; CRP, cAMP receptor protein; *cat*, structural gene for chloramphenicol acetyl transferase; bp, base pairs; kb, kilo bases; Tc, tetracycline

2. Experimental

Routine chemicals were purchased from Merck (Darmstadt). Acrylamide and *N,N*-methylene-bis-acrylamide were from Serva (Heidelberg), [^{3}H]UTP from The Radiochemical Centre (Amersham) and restriction endonucleases from Boehringer GmbH (Mannheim).

Plasmid pAC184 was isolated according to [7], and the 1.5 kb *Hin*dIII/*Eco* RI fragment recovered from agarose gels according to [8]. RNA polymerase was purified according to known procedures [9] and re-chromatographed on Bio-gel A1.5 M to ensure complete removal of contaminating nucleases. RNA synthesis was performed in 20 μl reaction mixtures containing 40 mM Tris--HCl (pH 7.9), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 100 mM NaCl, 0.1 mg/ml gelatin, 100 μM each ATP, GTP and CTP, 5 μCi [^{3}H]UTP (43 Ci/mmol), 2 μg RNA polymerase and 1 μg DNA (total plasmid), or 1 μg RNA polymerase and 0.5 μg DNA (*Hin*dIII/*Eco* RI fragment). When transcription was performed in presence of cAMP and CRP, these were added at 1 mM and 0.5 μg, respectively, and the order of addition of components was as in [10]. After 10 min at 37°C, reactions were terminated by adding 100 μl ice-cold transcription buffer, and RNA was extracted according to [10]. Electrophoresis of RNA transcripts on 4% acrylamide/7 M urea gels was as in [11], and fluorography according to [12].

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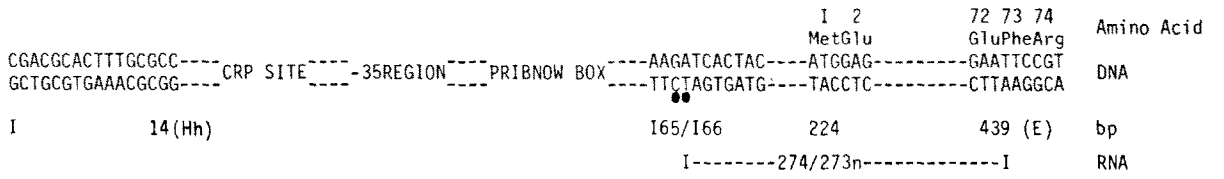
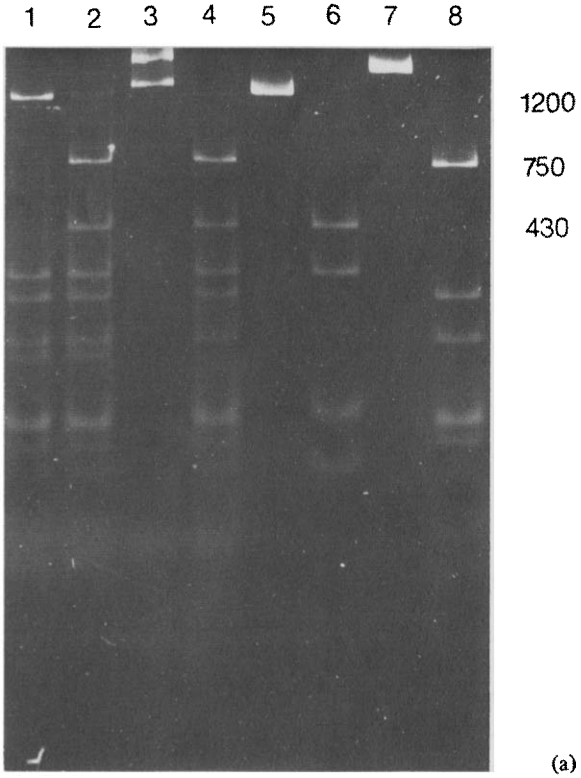


Fig.1. Structural features of the *cat* operon. Only those regions relevant to this work, i.e., from the *Hha* I site (14Hh) to the *Eco* RI site (439E) are indicated, because a detailed analysis is in [6]. The circles at bp 165, 166 are believed to be site of mRNA chain initiation. *Eco* RI cuts the DNA at a position corresponding to between amino acid residues 72 and 73, giving rise to the 274/273 nucleotide mRNA fragment indicated. This figure has been included with the authors' permission.

3. Results

Whilst the position of the *cat* gene on pAC184 has been established [5], its orientation has been in doubt. However, the *cat* gene has been sequenced [6,13], and a portion of this sequence, relevant to the presented work, is shown in fig.1. The DNA sequence of the *cat* gene and the amino acid sequence of chloramphenicol acetyl transferase [14] indicate that the promoter resides on a 425 bp fragment produced by double digestion of pAC184 with the restriction endonucleases *Eco* RI and *Hha* I. From this promoter transcription will proceed leftwards towards the *Eco* RI site.

As shown in fig.2a, *Hha* I digestion of pAC184



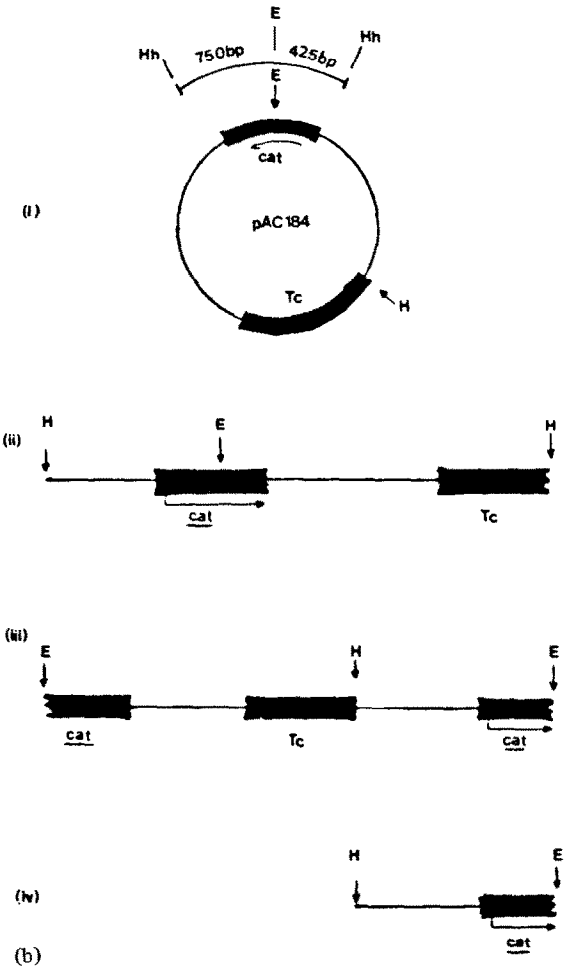


Fig.2. (a) Restriction analysis of pAC184. Digests are as follows: (1) *Hha* I; (2) *Eco* RI/*Hha* I; (3) *Hin*dIII/*Eco* RI; (4) *Hin*dIII/*Eco* RI/*Hha* I; (5) purified 1.5 kb *Hin*dIII/*Eco* RI fragment; (6) *Hha* I digest of 5; (7) purified 2.5 kb *Hin*dIII/*Eco* RI fragment; (8) *Hha* I digest of 7. (b) DNA templates transcribed in vitro. (i) Supercoiled pAC184; also shown are the *Eco* RI (E), *Hin*dIII (H) and *Hha* I (Hh) sites used to determine the orientation of the *cat* gene; (ii) *Hin*dIII-cut pAC184; (iii) *Eco* RI-cut pAC184; (iv) purified 1.5 kb *Hin*dIII/*Eco* RI fragment. In all cases, the arrow indicates the direction of transcription.

generates besides smaller fragments a 1200 bp fragment (track 1) which is subsequently cleaved by *Eco* RI into fragments of ~750 bp and 430 bp (track 2). Double digestion of pAC184 with *Hin*dIII and *Eco* RI produces fragments of ~1.5 kb and 2.5 kb (tracks 3,5,7); when the purified 1.5 kb *Hin*dIII/*Eco* RI fragment is cleaved with *Hha* I, a fragment of 430 bp is generated (track 6). No similar fragment is produced when the purified 2.5 kb *Hin*dIII/*Eco* RI fragment is cleaved with *Hha* I (track 8), but the 750 bp fragment is obtained. Both fragments are also observed in a *Hin*dIII/*Eco* RI/*Hha* I triple digest (track 4). These data thus demonstrates that the 425 bp *Eco* RI/*Hha* I fragment, predicted from the DNA sequence, can be generated from the small *Hin*dIII/*Eco* RI fragment. As predicted, this fragment carries the promoter for the *cat* gene.

Fig.2b shows the forms of pAC184 used as templates for in vitro transcription studies: (i) supercoiled; (ii) linearised with *Hin*dIII (iii) linearised with *Eco* RI; (iv) the purified 1.5 kb *Hin*dIII/*Eco* RI fragment. Further consideration of the DNA sequence predicts that transcription of forms (iii) and (iv) should produce an incomplete transcript of the *cat* gene ~270 nucleotides long if transcription proceeds towards the *Eco* RI site. Such transcript is indeed obtained when either *Eco* RI-cut pAC184 or the purified 1.5 kb *Hin*dIII/*Eco* RI fragment are used as templates (fig.3, tracks 2,4); furthermore, when these templates are transcribed in presence of cAMP and CRP, the rate of synthesis of this transcript is markedly enhanced (fig.3, tracks 1,3). Densitometer scans of the autoradiograms have estimated the enhancement at 15-fold with *Eco* RI-cut pAC184 and 6-fold with the 1.5 kb *Hin*dIII/*Eco* RI fragment as templates, also presented in fig.3. Since the *cat* gene is

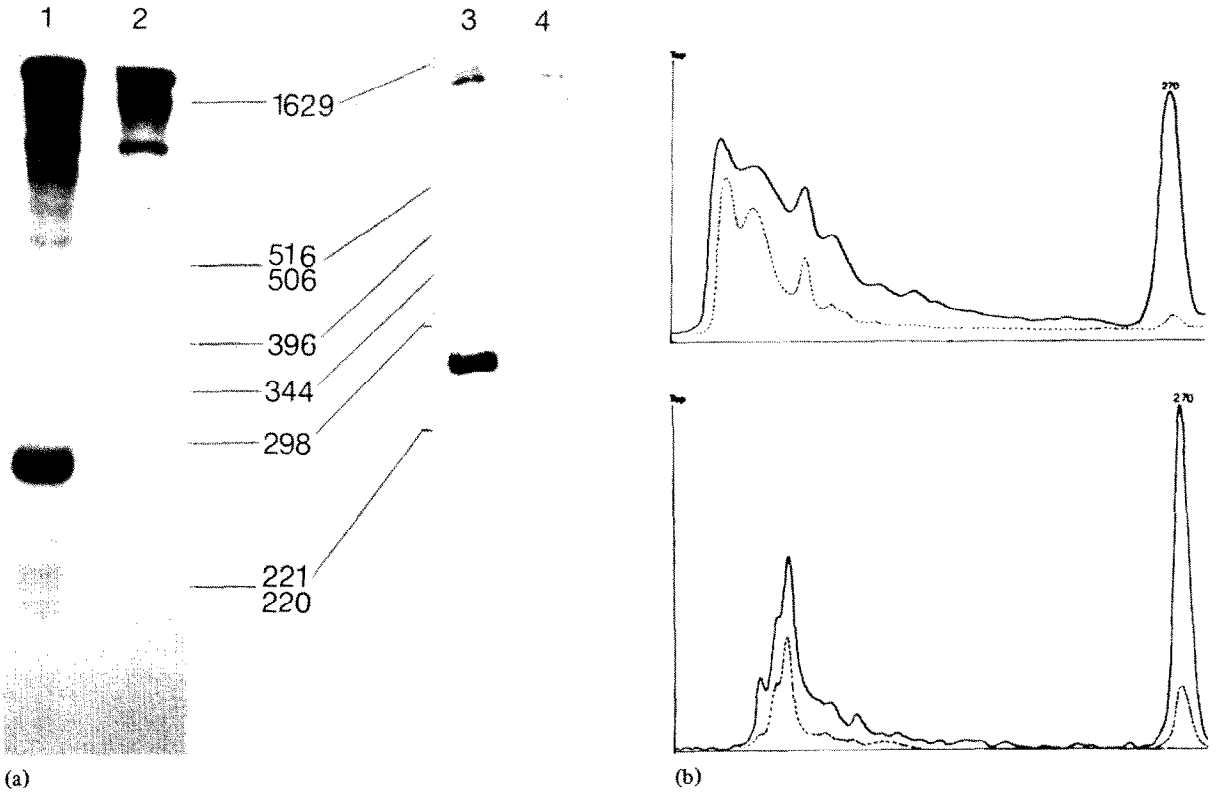


Fig.3. In vitro transcription of enzymatically cleaved pAC184. (a) Autoradiogram of RNA gel: (1) *Eco* RI-cut pAC184, +cAMP and CRP; (2) *Eco* RI-cut pAC184, –cAMP and CRP; (3) 1.5 kb *Hin*dIII/*Eco* RI fragment, +cAMP and CRP; (4) 1.5 kb *Hin*dIII/*Eco* RI fragment, –cAMP and CRP. (b) Densitometer scans of autoradiograms. The upper scan represents transcription of *Eco* RI-cut pAC184 and the lower scan of the *Hin*dIII/*Eco* RI fragment. (– – –) –cAMP and CRP; (——) +cAMP and CRP. Molecular weight markers are ^{32}P end labelled, thermally denatured DNA fragments produced by *Hin*fI digestion of plasmid pBR322.

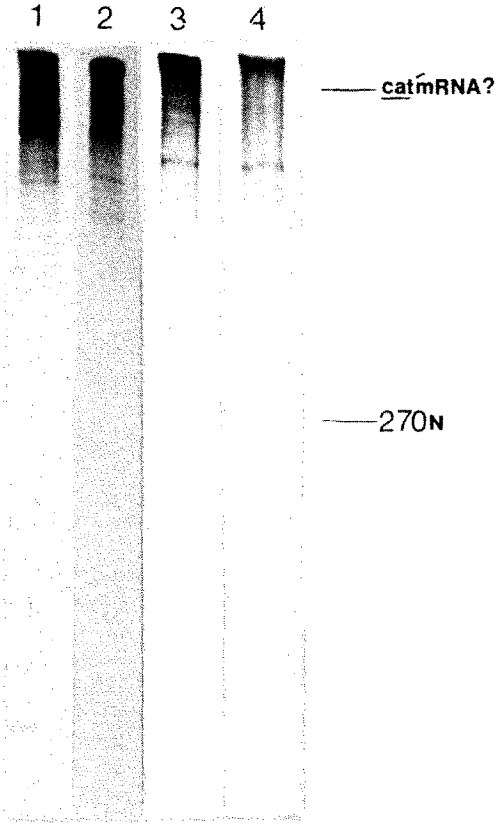


Fig.4. In vitro transcription of intact *cat* gene of pAC184: (1) supercoiled pAC184, –cAMP and CRP; (2) supercoiled pAC184, +cAMP and CRP; (3) *Hin*dIII-cut pAC184, +cAMP and CRP; (4) *Hin*dIII-cut pAC184, –cAMP and CRP. The possible *cat* transcript is indicated, as well as the position of the 270 nucleotide transcript.

catabolite sensitive [4], we believe the 270 nucleotide transcript to be representative of that part of the *cat* gene transcribed from the promoter towards, and terminating at, the *Eco* RI site.

If this 270 nucleotide transcript is representative of a portion of the *cat* mRNA, it should not be produced when either supercoiled or *Hin*dIII-cut pAC184 are used as templates, because in both cases the *cat* gene would be uninterrupted. Transcription of these templates should produce an mRNA species corresponding to the entire *cat* gene, ~750 nucleotides long. As can be seen from fig.4, no 270 nucleotide transcript is observed when forms (i) and (ii) of pAC184 are used as templates, neither in absence nor in presence of cAMP and CRP. The size of the complete *cat* transcript and the gel system, we have employed, make detection of the intact transcript difficult; however, in the case of *Hin*dIII-cut pAC184, a high molecular weight RNA species is produced and subject to stimulation with cAMP and CRP (fig.4, tracks 3,4). Without reliable molecular weight markers we cannot ascribe this transcript to the intact *cat* mRNA. It should be obvious, however, that the 270 nucleotide transcript, shown in fig.3, is not obtained when DNA spanning the entire *cat* gene is transcribed.

As well as the similarity between the size of transcript we have obtained and that predicted from the DNA sequence when transcribing from the promoter to the *Eco* RI site, proof that the 270 nucleotide transcript represents a portion of *cat* mRNA has been supplied by independent transcription studies of pAC184 by electron microscopy. This analysis has shown that transcription of the *cat* gene of pAC184 initiates some 6% of its genome length to the right of and proceeds towards the *Eco* RI site (D. Stüber and H. Bujard, personal communication). This distance, ~250 bp, is in good agreement with the size of transcript, we have obtained.

4. Discussion

The mechanism whereby cAMP and CRP control certain operons is still poorly understood. One approach to gain deeper insight to the process would be to isolate the appropiate control regions and analyse the binding of RNA polymerase and CRP to them. This has been done with the *lac* and *gal* operons, and apparently characteristic DNA sequences have been detected [15,16].

Here we have begun to analyse another catabolite sensitive system, the *cat* gene. This is expressed constitutively in *E. coli*, and it is unclear why its expression is subject to catabolite repression at all. By analysis of RNA transcripts synthesized either on the linearised plasmid pAC184 or on a 1.5 kb *Hin*dIII/*Eco* RI fragment thereof, we could show that transcription of the *cat* gene initiates 270 bp to the right of the *Eco* RI site, that it proceeds counterclockwise and is strongly influenced by cAMP and CRP. Determination of the transcription initiation site permits analysis of the DNA sequence recognised by CRP, and experiments of this nature are presently underway.

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