Unwinding of duplex DNA during transcription initiation at the *Escherichia coli* galactose operon overlapping promoters

Bernard Chan*, Stephen Minchin and Stephen Busby

School of Biochemistry, University of Birmingham, PO Box 363, Birmingham B15 2TT, UK

Received 19 April 1990

We have used potassium permanganate as a probe to detect DNA duplex unwinding in vitro, in open complexes between *E. coli* RNA polymerase and DNA fragments carrying the *E. coli* galactose operon regulatory region. This zone contains 3 overlapping promoters which specify transcription initiation at 3 distinct startpoints. We have used mutant *gal* derivatives carrying different single point mutations, each of which allows initiation from only one of the 3 start sites. This has allowed us to compare duplex unwinding in open complexes at the 3 different promoters, and to show that the extent of the unwinding is similar in each case. Further, the pattern of DNA modification by potassium permanganate suggests a model for discrimination between the upper and lower strands. Finally, we show that DNA modification by potassium permanganate at the *gal* promoters is the same in vivo as in vitro.

Overlapping tandem promoter; RNA polymerase; Open complex; Permanganate footprinting; DNA unwinding; *Escherichia coli*

1. INTRODUCTION

When RNA polymerase initiates transcription at a promoter, the DNA duplex around the transcription start site must be unwound to allow the template strand to pair with nucleotides held in the active site of the enzyme. This unwinding can be detected using chemical probes that are specific for single-strand DNA (e.g. [1,2]). In this paper we describe experiments in which we have used potassium permanganate, both in vitro and in vivo, as a reagent to detect unwinding when *E. coli* RNA polymerase initiates transcription at the *E. coli* galactose operon regulatory region. This region is unusual as it contains two overlapping but distinct promoters, *P1* and *P2*, that specify transcription initiation from start sites *S1* and *S2*, respectively, which are staggered by 5 base pairs [3]. Crucial evidence that these promoters are distinct comes from studies with mutations that specifically inactivate either *P1* or *P2*, leaving the alternative promoter *P2* or *P1*, respectively, unaffected [4–6]. In this study we have exploited these mutations to probe DNA unwinding during transcription initiation at *S1* or *S2*.

Recently, whilst studying the effects of a mutation that simultaneously inactivated both *P1* and *P2*, we discovered a third promoter, *P3*, which specifies transcription initiation from a site, *S3*, located 14 base pairs downstream of *S1* [7]. *GalP3* is a weak promoter in vitro, and in vivo expression is very low [5,7]. Here we show that the DNA duplex around *S3* is unwound during transcription initiation at *P3* and that the pattern of permanganate-sensitive bases is qualitatively similar to those found at *P1* and *P2*.

Correspondence address: S. Busby, School of Biochemistry, University of Birmingham, PO Box 363, Birmingham B15 2TT, UK

* *Present address:* Delta Biotechnology, Castle Court, 59 Castle Boulevard, Nottingham NG7 1FD, UK

2. MATERIALS AND METHODS

The *gal* operon regulatory region was cloned on a 144 base pair DNA fragment between the *Eco*RI and *Hin*dIII sites of pBR322 as previously described [8]. The *gal* sequence, numbered with *S1* as +1 is shown in Fig. 1. The fragment carried a GC to TA transversion or a GC to AT transition at position −19 (*p19T* or *p19A*), a GC to AT transition at −14 (*p14*), or a TA to CG transition at −12 (*p12*). The isolation and characterisation of these mutations have been described previously [5,7,9]. *Pst*I–*Hin*dIII fragments containing these gal sequences were purified as before and, using standard methods [10], labelled at the *Hin*dIII end, either on the lower strand using [γ-^{32}P]ATP and T4 polynucleotide kinase, or on the upper strand using [α-^{32}P]dATP and Klenow fragment.

In footprinting experiments in vitro, labelled fragments were incubated with purified *E. coli* RNA polymerase exactly as before [7,11]. Routinely 1 μl of freshly prepared 200 mM potassium permanganate was added to 20 μl samples and, after 4 min at 37°C and alcohol precipitation, the DNA was treated with piperidine which causes strand scission at the sites of modification [12,13]. Labelled fragments generated in this way were resolved on sequence gels and detected by autoradiography precisely as before [14], using Maxam-Gilbert sequence reactions to calibrate the gels. In the in vivo experiment (Fig. 5), M182 cells carrying plasmid containing the *gal* promoter sequence were grown in minimal medium with fructose [4] to an absorbance of 0.5 at 600 nm. 270 μl of 0.37 M stock potassium permanganate was then added to a 10 ml sample of cells and, after 4 min the cells were harvested and plasmid DNA was purified (in some cases 40 μl of 50 mg/ml rifampicin was also added immediately prior to the permanganate). In vivo-modified plasmid DNA was linearised with *Bst*EII, which cuts uniquely at +38 [7], labelled on the

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

lower strand as described above and then restricted with *Hin*dIII, which cuts uniquely at +48. After piperidine treatment, permanganate-induced modification of *gal* sequences downstream of position +38 was revealed by gel analysis. In the experiment with supercoiled plasmid DNA in vitro (Fig. 5), open complexes were formed and subjected to potassium permanganate treatment as before. The DNA was then purified by phenol extraction and the sites of modification were located as in the in vivo experiment.

3. RESULTS AND DISCUSSION

3.1. *Permanganate modification of open complexes at* galP1, P2 *and* P3 *in vitro*

Fig. 1 shows the nucleotide sequence around the *gal* promoters together with the mutations that we have exploited in this study. With the *p19T*, *p14* and *p12* mutations, we have previously shown that RNA polymerase initiates transcription in vitro at *S1*, *S2* and *S3*, respectively [5,7]. Fig. 2 shows the pattern of modification by potassium permanganate, on both the upper and lower strands, when RNA polymerase forms open complexes at DNA fragments carrying the *gal* promoter region with each of these mutations. The results are presented schematically in Fig. 3. When polymerase initiates transcription at *S1*, thymines are modified on the lower strand at positions +2, +1, −5 and −11 whilst on the top strand modification is found at positions +3, +2, −1 and −3 (Figs 2 and 3, a: *galp19T*). As permanganate principally reacts with bases in unstacked DNA [12,13], we can deduce that the region of unwinding in the open complex runs from the upstream end of the −10 hexamer sequence (5′ TATGGT 3′) to just downstream of the transcription start. Note that modification is contingent on the addition of RNA polymerase (compare − and + lanes in Fig. 2). A similar situation is found when RNA polymerase initiates at *P2* (Figs 2 and 3, c: *galp14*): again the unwound region runs from the upstream end of the −10 sequence (5′ TATACT 3′) to just downstream of the start site at position −5. With initiation at *P3*, although this promoter is weak both in vitro and in vivo, clear polymerase-dependent permanganate-induced base modification is seen from just downstream of the transcription start site at +14 to the upstream end of the −10 sequence 5′ TACCAT 3′ (Figs 2 and 3, d: *galp12*). Our results show that, according to which promoter is operational, a different, approximately 14 base pair stretch of sequence, is opened.

During the mutagenesis of the *gal* operon regulatory region we isolated a second mutation at position −19, *p19A* [5,15] (see Fig. 1). Transcription of *gal* promoter fragments carrying this mutation starts at both *S1* and *S3* [15]; Figs 2 and 3, b show the location of base modification by permanganate at open complexes with RNA polymerase. The pattern of the modification clearly comprises signals from polymerase at both *P1* and *P3* (compare results in Figs 2 and 3, b, *p19A*, with a, *p19T*, and d, *p12*). Presumably with this promoter sequence, *P1* and *P3* are about equally active and polymerase binds to either one or the other promoter on any fragment: *P3* can thus be active in the absence of the p12 mutation. Furthermore, in experiments with *galp19T*, some faint bands, indicative of a trace amount of open complex formation at *P3*, were seen when gels were overexposed (see, for example, Fig. 2, a: faint bands are detected downstream of +1 on the upper strand gel). We can conclude that the nature of the mutation at position −19 fixes the relative activities of *P1* and *P3*.

From the compilation of results, shown in Fig. 3, it is clear that the pattern of bases that are sensitive to permanganate in open complexes at *P1*, *P2* or *P3* is the same, permanganate preferentially modifying unstacked thymines. However, whilst the thymines in the unwound region on the lower strand are modified, we find less modification of the bases in the −10 hexamer on the upper strand, although this sequence is clearly melted [1,2,16]. This is most clear in the case of *galp19T* where no modification of upper strand thymines at positions −10, −6 and −7 is found (Fig. 2, a). A simple explanation for this is illustrated by the sketch in Fig. 4. It is known that the −10 region is crucial for sequence-dependent recognition of promoters and it has been suggested that the sigma subunit binds to this region after unwinding [17]. We suggest that RNA polymerase binds to the upper unwound strand in the −10 region thus protecting these bases

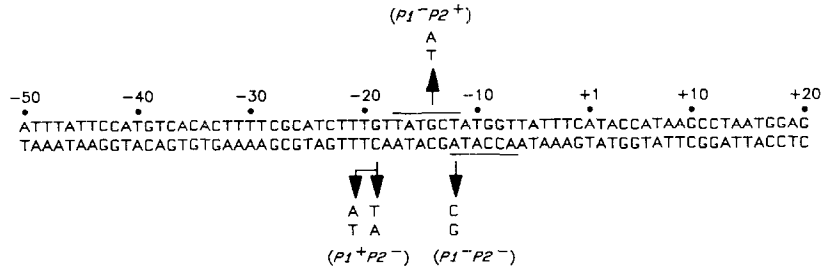

Fig. 1. Nucleotide sequence of the *gal* promoter region. The sequence is numbered with the *galP1* transcription start site as +1 and the −10 hexamer sequences corresponding to *galP1* and *galP2* (5′ TATGGT 3′ and 5′ TATGCT 3′, respectively) are overlined. The locations and nature of the point mutations discussed in the text are shown together with their effects on *P1* and *P2* activity.

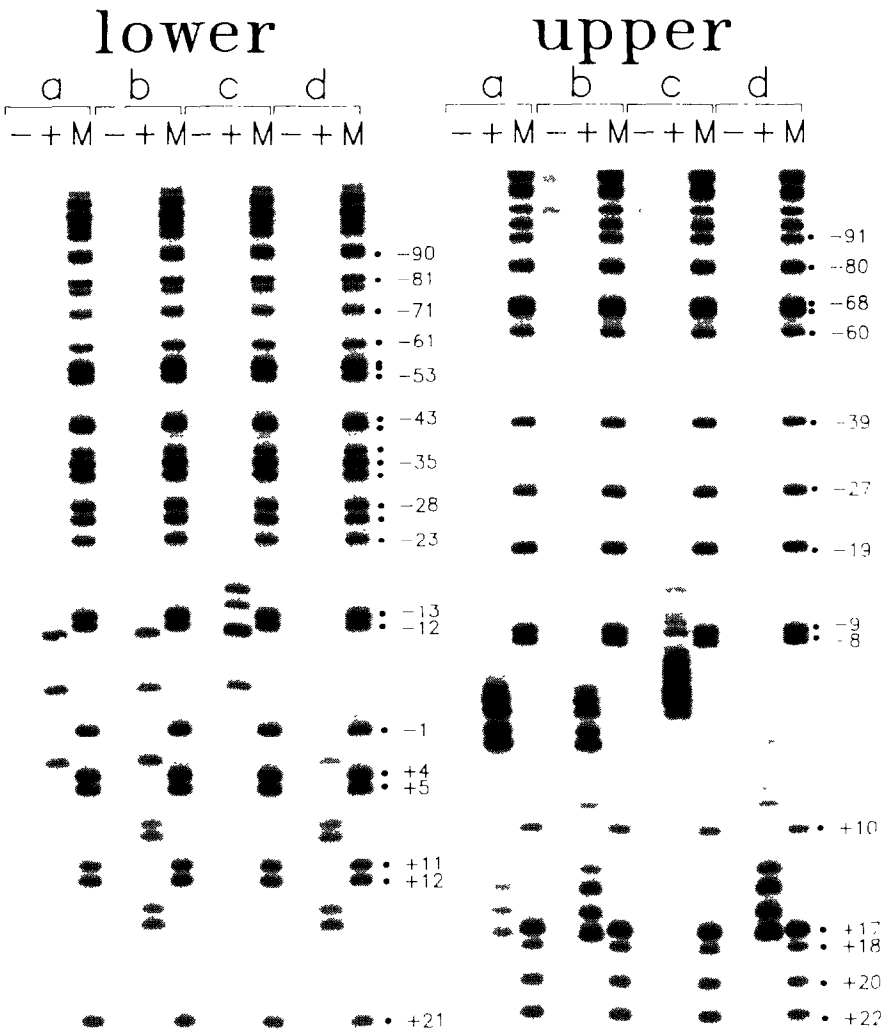

Fig. 2 Modification of *gal* promoter sequences by permanganate. The figure shows autoradiograms of gels to analyse the sites of base modification by permanganate at the *gal* promoter region. The fragments were labelled on the lower or upper strands at the *Hin*dIII site and carried the *p19T, p19A, p14* or *p12* mutations in tracks indicated by a, b, c and d, respectively. For each experiment, modification was performed in the absence (−) or presence (+) of RNA polymerase and the gel was calibrated from G-specific sequence reactions on labelled fragments (M). The calibrations shown are with respect to the *P1* start as in Fig. 1

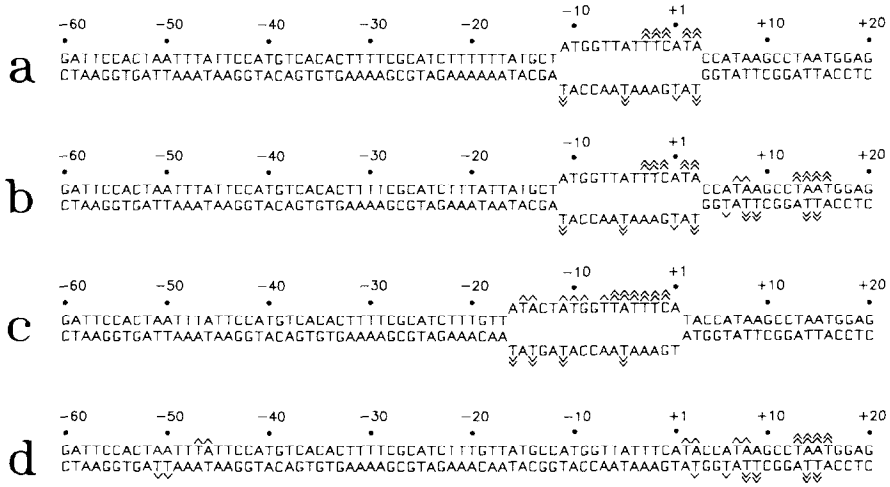

Fig. 3. Schematic representation of permanganate-modified bases. The figure shows the pattern of modification in open complexes at *galp19T* (a), *galp19A* (b), *galp14* (c) and *galp12* (d). Double and single carets represent stronger and weaker modification of positions and the separated strands illustrate the likely zone of base unstacking at *galP1* and *P2*.

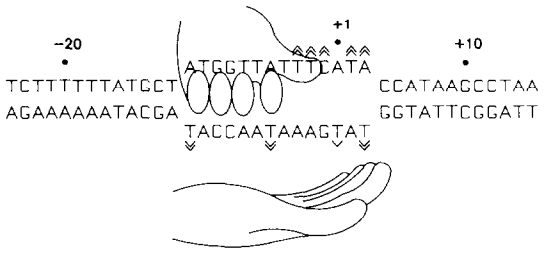

Fig. 4. Open complex formation at *galP1*. The figure is a speculative suggestion for how RNA polymerase interacts with zone around the −10 region at *galP1*. RNA polymerase is drawn as a pair of hands: one hand specifically recognises the upper strand sequence of the −10 hexamer and binds to it, protecting the bases from modification by permanganate. The other hand holds the lower strand in an open conformation, presumably setting it up for base pairing to incoming nucleotides and the 5′ end of the growing RNA chain. The carets locate the sites of base modification by permanganate in the open complex.

against modification by permanganate. Presumably the lower strand needs to be free in the active site of polymerase in order to base pair with incoming nucleoside triphosphates and, thus, it is accessible to permanganate. Interestingly, Jeppesen and Nielsen [18] recently reported data similar to ours with the *deoP1* promoter: they concluded that sequence from positions −12 to +3 was unwound in open complexes and that some of the bases in this region were protected from modification by specific protein contacts or by a fixed DNA conformation.

3.2. *Permanganate modification of the* gal *promoter region on supercoiled DNA in vitro and in vivo*

To check that the organisation of open complexes between the *gal* promoters and RNA polymerase was not altered by DNA supercoiling, we repeated the experiment using purified supercoiled plasmid carrying *galp19T*. Panel I of Fig. 5 shows that the pattern of modified bases on the lower strand in open complexes at *galP1* is substantially the same as in the experiment with linear fragments (cf. Fig. 2, a). Next we repeated the experiment to probe the reactivity of *galp19T* sequences towards permanganate in vivo, as described in section 2. Panel II of Fig. 5 shows that, again, the pattern of modified bases is qualitatively similar to that found with linear fragments in vitro. Comparison of

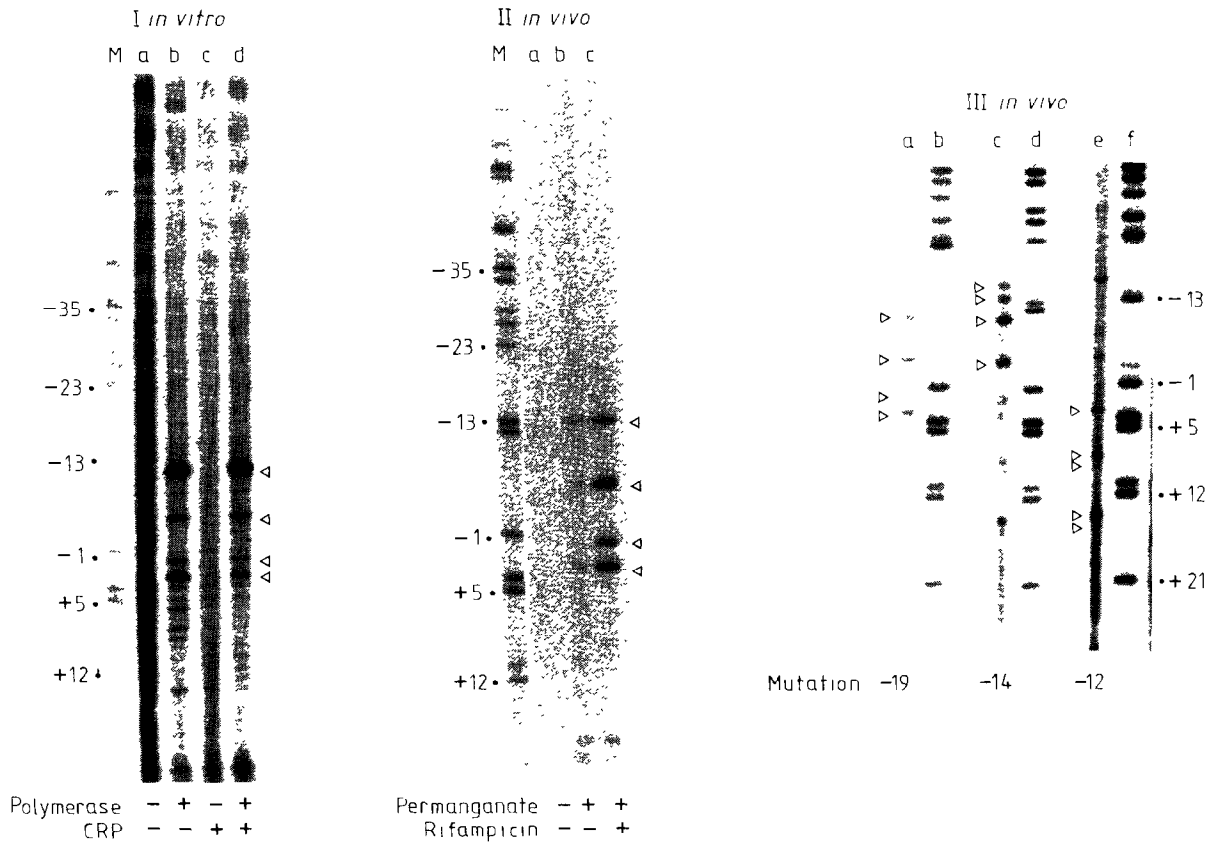

Fig. 5. Modification of *gal* promoter sequences in supercoiled DNA in vitro and in vivo. Panel I shows the result of an experiment in which supercoiled purified *galp19T* DNA was probed with permanganate in the presence of different combinations of RNA polymerase and cAMP-CRP as shown in lanes a–d. The gel is calibrated with a G-specific sequence reaction (M). In panel II growing cells carrying *galp19T* were treated with permanganate and rifampicin as shown in lanes a–c. The locations of permanganate-sensitive bases were found by reference to the calibration, M. In panel III, growing cells carrying *galp19T*, *galp14* or *galp12*, as shown, were treated with permanganate and sites of modification were deduced from tracks a, c and e using the calibrations in lanes b, d and f.

lanes b and c in this panel shows that, as found by others [19], the addition of rifampicin to the culture immediately prior to permanganate, increases the amplitude of the signals. Panel III of Fig. 5 shows an experiment where we compared the reactivity of *galp19T, p14* and *p12* in vivo. The results clearly show that open complexes form at *galP1, P2* and *P3*, respectively: again, the patterns of reactivity are similar to those found with linear fragments. Thus, we can conclude that open complexes probed in vitro with linear fragments, correspond to intermediates which form in vivo.

Acknowledgements: This work was supported by grants from the UK SERC (Grant GRE 45151) and the Wolfson Foundation.

REFERENCES

[1] Siebenlist, U., Simpson, R. and Gilbert, W. (1980) Cell 20, 269–281.
[2] Kirkegaard, K., Buc, H., Spassky, A. and Wang, J. (1983) Proc. Natl. Acad. Sci. USA 80, 2544–2548.
[3] Musso, R., Di Lauro, R., Adhya, S. and De Crombrugghe, B. (1977) Cell 12, 847–854.
[4] Busby, S. and Dreyfus, M. (1983) Gene 21, 121–131.
[5] Bingham, A., Ponnambalam, S., Chan, B. and Busby, S (1986) Gene 41, 67–74.
[6] Kuhnke, G., Krause, A., Heibach, C., Gieske, U., Fritz, H.-J. and Ehring, R. (1986) EMBO J. 5, 167–173.
[7] Ponnambalam, S., Spassky, A. and Busby, S. (1987) FEBS Lett. 219, 189–196.
[8] Ponnambalam, S. and Busby, S. (1987) FEBS Lett. 212, 21–27.
[9] Bingham, A. and Busby, S. (1987) Mol. Microbiol. 1, 117–124.
[10] Sambrook, J., Fritsch, E. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn, Cold Spring Harbor, NY.
[11] Spassky, A., Busby, S and Buc, H. (1984) EMBO J. 3, 43–50.
[12] Borowiec, J., Zhang, L., Sasse-Dwight, S. and Gralla, J. (1987) J. Mol. Biol. 196, 101–111.
[13] O'Halloran, T , Frantz, B., Shin, M., Ralston, D. and Wright, J. (1989) Cell 56, 119–129.
[14] Chan, B. and Busby, S. (1989) Gene 84, 227–236.
[15] Chan, B. (1989) PhD Thesis, University of Birmingham.
[16] Spassky, A., Rimsky, S., Buc, H. and Busby, S. (1988) EMBO J. 7, 1871–1879.
[17] Helmann, J. and Chamberlin, M. (1988) Annu. Rev. Biochem. 57, 839–872.
[18] Jeppesen, C. and Nielsen, P. (1989) Nucleic Acids Res. 17, 4947–4956.
[19] Sasse-Dwight, S. and Gralla, J. (1989) J. Biol. Chem. 264, 8074–8081.