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Identifying cloned *Helicobacter pylori* promoters by primer extension using a FAM-labelled primer and GeneScan® analysis

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Abstract

The transcriptional start sites of 27 promoters in *Helicobacter pylori* strain 4187E have been successfully identified using a non-radioactive primer extension protocol. The technique involves reverse transcribing mRNA with a sequence-specific FAM-labelled primer. The length of the FAM-labelled cDNA primer extension product can be analysed on a standard DNA sequencer using GeneScan® software. This information can be used in conjunction with DNA sequencing data to identify the transcriptional start site of a promoter. Total bacterial RNA produced more specific primer extension products with stronger FAM signals than a population enriched for mRNA. Using this technology, it is not necessary to complete the DNA sequencing reactions in parallel with the primer extension experiments. The FAM-labelled primer extension products do not require a PCR amplification step prior to analysis on a sequencing gel, and no phenol/chloroform purifications are required at any stage of the procedure. Fluorescent-based primer extension methods have obvious advantages over the conventional radioactive protocols, and this report extends the currently used methodologies in this field.

Keywords: Promoter identification; Transcriptional start site; GeneScan®; FAM; Fluorescent primer; Primer extension

1. Introduction

In order to understand how an organism regulates its gene expression at the transcriptional level, it is necessary to locate and characterise the promoter regions of the genes involved. Promoters should be functionally defined as sequences that direct initiation of transcription. A region of DNA can be characterised as a promoter with reasonable certainty if it can be shown to direct the transcription of mRNA in vivo, or in vitro using a functional assay. Therefore, the experimental identification of the transcriptional start site (+1) is the first step in identifying the promoter region. The long-term aim of this research is to isolate and characterise *H. pylori* promoters that are functional in *Escherichia coli*, and this paper describes the

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sequence and transcriptional start site for a *H. pylori* promoter using a fluorescence-based primer extension protocol.

In prokaryotes, the processes of transcription and translation are coupled. The mRNA transcript undergoes little processing or modification, and is used directly to synthesize proteins during translation.

Isolating and working with mRNA from eukaryotes is easier in comparison to prokaryotes, as eukaryotic mRNA is polyadenylated and is considerably more stable than prokaryotic mRNA. Prokaryotic mRNA has a half-life of only several minutes, whereas eukaryotic mRNA can remain stable for an hour or more (Rauhut and Klug, 1999; Takayama and Kjelleberg, 2000). The poly(A) tail of eukaryotic mRNA facilitates the easy and efficient isolation of this mRNA population through binding to an oligo(dT) probe or matrix. In contrast, the isolation of intact, stable bacterial mRNA is a significantly more difficult and time-consuming process.

The two conventional methods used to determine transcriptional start sites are S1 nuclease mapping and radioactive primer extension experiments. S1 nuclease mapping is frequently used to locate the transcriptional start sites of bacterial promoters, including recent work mapping promoters of *Escherichia coli*, *Neisseria meningitidis* (Delany et al., 2004) and *Mycobacterium tuberculosis* (Master et al., 2001). A range of *E. coli* promoters have been identified using this technique, including the Mg^{2+} responsive promoters of the *pho*PQ, *mgt*A and *mgr*B genes (Kato et al., 1999), the promoters of the *cai* and *fix* operons (Buchet et al., 1998), and the iron-regulated promoter of the *E. coli* plasmid ColV-K30 (Bindereif and Neilands, 1985). Each of these methods has required between 20 and 100 μg of total RNA per S1 nuclease protection assay.

The majority of the currently available literature on primer extension protocols tends to focus on conventional ^{32}P- or ^{35}S-based methods of locating transcriptional start sites. These methods are potentially hazardous, time consuming, and require laboratories specifically designated for radioisotope research.

Limited information exists in the literature describing fluorescent-based primer extension protocols. Several of these publications have described methods which show some similarities to the research presented here. Yamada et al. (1998) used 0.1–10 μg of total RNA, 1 pmol of fluorescein isothiocyanate (FITC)-labelled primer and 20 U of RNase inhibitor in a final volume of 20 μL. The primer extension reaction was commenced by the addition of 5 U of Avian Myeloblastosis Virus (AMV) reverse transcriptase (RT) and samples were incubated at 50 °C for 60 min. Carrier tRNA was added when the total RNA was less than 1 μg, and the samples were subjected to a phenol extraction, followed by ethanol precipitation. The FITC-labelled cDNA products were analysed on a DNA sequencer. Altermann et al. (1999) described a Nonradioactive Automated Primer Extension technique which they termed NAPE. They used a primer containing a 5′ -IRD800 fluorescent label, 5–10 μg of total RNA, 0.1–1.5 μM of primer and 20 U of AMV RT. This method comprised a single primer extension step for 90 min, followed by RNase A digestion and concentration of nucleic acids by precipitation with spermine tetrahydrochloride. The fluorescent cDNA products were analysed on a DNA sequencer. Fekete et al. (2003) described a technique known as Fluorescently Labelled Oligonucleotide Extension (FLOE). The FLOE protocol used 2.5 pmol of primer containing a 5′ -FAM label, 7 μg or 0.7 μg of total RNA and 15 U of AMV RT. The FAM-labelled cDNA products were cartridge purified and separated on capillary columns on an ABI PRISM® 3100 Genetic Analyser. The size of the primer extension products was determined using the Scanalyze program (version 0.4), which has subsequently been renamed Chromagna (M.J. Miller, personal communication).

The major features of the primer extension method described in this report that set it apart from published methods are the comparison of total bacterial RNA versus mRNA, the inclusion of an enrichment step incorporating a second reverse transcription reaction, and the fact that the primer extension products do not need to be passed through a column-based purification procedure before analysis on a standard DNA sequencing gel. The need for an alternative method of primer extension promoter identification that is simple, sensitive, cost-effective, non-radioactive and suitable for use with total bacterial RNA has its obvious advantages. This research presents the development and optimisation of an alternative method of locating transcriptional start sites of bacterial promoters.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions and MIC determination

*Helicobacter pylori*4187E is a clinical isolate (McColm, 1997) which was kindly provided by Dr. B.J. Appelmelk (Vrije University, Amsterdam). *E. coli* DH5α was purchased from Invitrogen Australia, and plasmid pKK232-8 (Brosius, 1984) was purchased from Amersham Biosciences, Australia. Vector pKK232-8 contains a promoterless chloramphenicol acetyltransferase (CAT) gene. *H. pylori* was grown on blood agar plates at 37 °C under microaerophilic conditions with 10% CO_2 and 95% humidity. *E. coli* cultures were grown at 37 °C in Luria Bertani (LB) broth with shaking or on LB agar plates. Where appropriate, *E. coli* cultures were supplemented with 10 μg/mL chloramphenicol (Cm), unless otherwise stated. Cm minimum inhibitory concentrations (MICs) were determined for each clone by replica plating neat and 1:10 dilutions of overnight cultures onto LB plates containing doubling dilutions of Cm from 640 to 5 μg/mL. Plates were incubated for 24–36 h at 37 °C.

2.2. DNA manipulations

All DNA manipulations were carried out as described by Sambrook and Russell (2001). Vector pKK232-8 was isolated from *E. coli* cultures using the QIAprep® Spin Miniprep Kit (Qiagen, Germany). Random fragments of *H. pylori* 4187E were cloned into the promoter-trap vector pKK232-8 using the restriction enzymes *Sau*3AI and *Hae*III, and promoter-containing transformants were selected for on LB plates containing 10 μg/mL Cm. Restriction enzymes were obtained from Promega. DNA sequencing primers were synthesized by Invitrogen Life Technologies, Australia. The primer for the primer extension reaction (PE+FAM) was 30 nucleotides in length, contained a 5′ 6-carboxyfluorescein (FAM) label and was synthesized by GeneWorks (Adelaide, Australia). The PE+FAM sequence (5′ -ATTTGTCCTACTCAAGCTTGGCTGCAGGTC-3′) was complementary to the region between the multiple cloning site (bases 177–212) and the beginning of the CAT gene (bases 269–928) of vector pKK232-8. The nucleotide sequence of pKK232-8 was obtained from GenBank (http://www.ncbi.nlm.nih.gov) (Accession Number U13859).

2.3. DNA sequencing

DNA sequencing of cloned promoters was performed using the ABI Prism® BigDye™ Terminators version 3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems, Foster City, CA). DNA sequencing products were analysed on an ABI 377 automated sequencer (Applied Biosystems).

2.4. Total RNA and mRNA isolation

In order to maximise production of the CAT mRNA transcript, *E. coli* cultures were grown to log-phase in LB broth containing Cm concentrations below the MIC of the clone. Two-hour log-phase cultures of the *E. coli* clones were set up using a 15% inoculum from an overnight culture. All clones were grown in 10 μg/mL Cm for the first hour, and then the Cm concentration was raised to either 50 μg/mL (MIC=80 μg/mL Cm), 100 μg/mL (MIC=160 or 320 μg/mL Cm) or 200 μg/mL (MIC=640 μg/mL Cm) and the cultures were grown for a second hour. Total RNA was isolated from log-phase *E. coli* cultures using the RNeasy® Protect Bacteria Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA was eluted in diethylpyrocarbonate (DEPC)-treated water, quantitated by determining the absorbance at OD_{260} and stored at −80 °C. Total RNA samples were enriched for mRNA using the MICROBExpress™ Bacterial mRNA Enrichment Kit (Ambion, Austin, TX), which removes the 16S and 23S ribosomal RNAs from the total RNA population. Enriched mRNA populations were eluted in DEPC water and stored at −80 °C.

2.5. Primer extension protocol

Although experiments showed that 10 to 40 μg total RNA gave successful results, primer extension reactions were routinely carried out where PE+FAM primer (final concentration 5–10 nM) was added to 20 μg total RNA in a 0.5-mL microcentrifuge tube and the final volume was adjusted to 20 μL using DEPC water. Samples were heated at 70 °C for 5 min before

being quenched on ice for at least 10 min. Tubes were subsequently incubated at 58 °C for 20 min and were allowed to cool to room temperature for 15 min.

First strand cDNA synthesis was performed using AMV RT enzyme and 5× AMV RT buffer (Promega) according to the manufacturer's instructions. The primer extension reaction was completed in two stages. After an initial reverse transcription step, all of the reagents were replenished and the samples underwent a second extension reaction. The initial primer extension reaction contained 1× AMV RT buffer, 1 mM dNTPs (Gibco, Life Technologies, USA), 40 U of Recombinant RNasin® Ribonuclease Inhibitor and 20–25 U of AMV RT enzyme (Promega). The final volume was adjusted to 30 μL using DEPC water and the samples were incubated in a 42 °C waterbath for 1 h. Enrichment of the initial reverse transcription reaction was achieved following the addition of 1× AMV RT buffer and 1.5 mM dNTPs. The samples were supplemented with an additional 40 U of Recombinant RNasin® Ribonuclease Inhibitor and 20–25 U of AMV RT enzyme. The final volume was adjusted to 40 μL with DEPC water, and the samples were incubated at 42 °C for a further 1 h. Following the addition of 10 ng RNase A (10 μL at 1 ng/μL), the samples were incubated at 37 °C for 30 min.

FAM-labelled cDNAs were allowed to precipitate for 15 min at room temperature following the addition of 0.1 volume of 3 M sodium acetate and 2.5 volumes of absolute ethanol. cDNA was pelleted by centrifugation at 25,000×*g* for 20 min and each pellet was washed with 70% ethanol before being air-dried and stored at −20 °C.

Electrophoresis was performed using an ABI 377 DNA Sequencer (Applied Biosystems) which had been pre-run at 1000 V until the gel temperature reached 51 °C. Each cDNA sample was dissolved in a solution consisting of 2.5 μL formamide (Promega), 0.5 μL GeneScan® −500 ROX™ internal lane standard (Applied Biosystems), and 2 μL of loading buffer (Applied Biosystems) per sample. Samples were heated to 94 °C for 3 min and a 5 μL aliquot of the sample was loaded into alternate lanes onto a 5% polyacrylamide-urea gel Long Ranger® gel (Bio-Whittaker Molecular Applications, Rockland, ME). The gel was electrophoresed for 2 h at a constant voltage of 3.0 kV. The DNA fragments were sized using the GeneScan® Analysis Software version 3.1 (Applied Biosystems), using Filter Set D.

3. Results and discussion

3.1. Total RNA versus mRNA

Transcriptional start sites for 27 cloned *H. pylori* promoters have been identified using this fluorescent-based primer extension protocol. Primer extension signals obtained using mRNA were weaker than those obtained using total RNA. Fig. 1 shows the electropherograms obtained for primer extension experiments on clone C1, producing a 281 base cDNA product, using (A) 20 μg total RNA and (B) 1 μg mRNA. The primer extension results for clone C1 (shaded blue peaks) clearly illustrate that total RNA gave a stronger FAM signal than mRNA, as indicated by the peak heights of the cDNA products (318 versus 110, respectively).

Bacterial cells in culture were stabilised using the Qiagen RNAprotect® Bacteria Reagent to ensure the RNA profile was not modified by the handling protocols, resulting in artifactual changes to gene expression profiles. Expression of individual genes may be induced or repressed during sample processing, which therefore affects the reproducibility of experimental data.

Enriching the total RNA population for mRNA using the MICROBExpress™ Bacterial mRNA Enrichment Kit was a time-consuming and expensive procedure that was not suitable for processing multiple samples at once. In addition, enrichment for mRNA did not improve the outcome of the primer extension experiments. The primer extension signals obtained using mRNA were weaker than those obtained using total RNA.

Primer extension products constructed using a FAM-labelled primer have a high level of precision and can allow quantitation of results. Each cDNA fragment contains a single 5′ fluorophore and therefore the mobility of all fragments is comparable. The product peaks tend to be narrow and the peak area is directly proportional to the number of cDNA molecules present in the sample.

It appears that the method used to isolate total RNA prior to mRNA enrichment affects the outcome

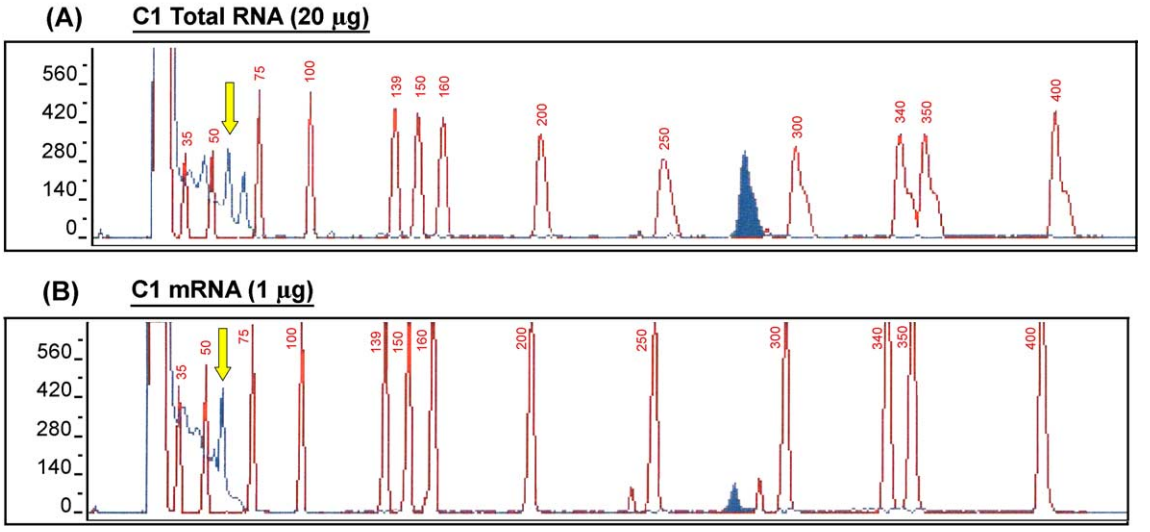

Fig. 1. Comparison of primer extension electropherograms using total and enriched mRNA. (A) Primer extension data obtained using 20 µg of total RNA. (B) Primer extension data obtained using 1 µg of enriched mRNA. The red peaks are the GeneScan®-500 ROX™ internal lane standards, and the size of each peak is shown (in base pairs). The shaded blue peak in each panel is the primer extension product (FAM-labelled cDNA) for clone C1. Peak height is a measure of fluorescence intensity and indicates strength of the FAM signal. Peak area is a measure of the relative amount of cDNA present in the sample. The peak height for the total RNA sample was 318 (Panel A), while the mRNA sample has a peak height of only 110 (Panel B). Lower peak heights correspond to a weaker FAM signal, as detected by the GeneScan® analysis software. More FAM-labelled cDNA product is produced from reverse transcription reactions using total RNA as template. As a result, all subsequent primer extension reactions were completed using total RNA. The common FAM signal of 58.61±0.21 bases (yellow arrow) was present in >95% of all samples.

of the MICROB*Express*™ procedure (Ambion Technical Services, personal communication). Column-based total RNA isolation kits, such as the RNeasy® Protect Bacteria Mini Kit used in these experiments, do not tend to isolate small RNA molecules such as tRNAs, 5S rRNAs and other small RNAs. The RNeasy® columns bind RNA fragments larger than approximately 200 nucleotides to the silica-gel membrane, with smaller RNA molecules passing through the column. As a result, the RNeasy® total RNA isolation protocol selectively enriches for mRNA molecules (RNeasy® Mini Handbook, Qiagen). However, we obtained lower mRNA yields from the MICROB*Express*™ kit than claimed by the manufacturer, despite successful mRNA isolation using the control RNA included in the kit. The discrepancies that were identified could have been due to the fact that this protocol was optimised for phenol-based total RNA isolation procedures which also isolate tRNAs, 5S rRNAs and other small RNA molecules, as well as mRNA species. Without these small RNA molecules, the total yields of mRNA from the MICROB*Express*™ kit are significantly lower than predicted as a proportion of the total input RNA.

3.2. Common FAM product

A common band was found in essentially all primer extension samples, including those samples which failed the primer extension reaction. This signal was detected in the blue (FAM) channel and was 58.61±0.21 bases long. Fig. 1 shows the common FAM product (yellow arrow) in the primer extension reaction for both the total RNA (panel A) and enriched mRNA (panel B) samples for Clone C1. The FAM signal does not appear to be a primer dimer, as analysis of the primer sequence revealed a single potential dimer of 32 bp. The fact that this product was consistently found in >95% of samples suggests that it is not an artifactual result. The presence of this band in all samples, each containing different fragments of cloned *H. pylori* DNA, suggests that this band is related to the vector sequence, the FAM label

or a common RNA species of the *E. coli* host. The PE+FAM primer does not show significant homology with any other regions of the vector sequence, and therefore, it is proposed that this finding could be related to the 5′ -FAM label on the oligonucleotide. This could be a fluorophore-mediated effect, related to dimerization of the FAM molecule rather than a sequence-related phenomenon.

3.3. Size of cDNA products detected

Primer extension products ranging from 66 bases to 372 bases were identified. Primer PE+FAM was 30 nucleotides in length, indicating the closest transcriptional start site was only 36 bases from the end of the primer. The limiting factor in obtaining cDNA fragments longer than 372 bases was the size of the cloned *H. pylori* DNA inserts, rather than due to specific technical challenges with the methodology. Several other fluorescent-based primer extension protocols have generated and detected cDNA products of 300 bases (Fekete et al., 2003), 560 bases (Yamada et al., 1998) and 570 bases (Altermann et al., 1999). There is no reason to believe that this technique could not be used to size cDNA fragments of 500 bases, or longer if an appropriate internal lane standard was used.

Fig. 2 contains a photograph of the primer extension gel for clone C3. The FAM-labelled cDNA product(s) are indicated by a blue arrow, and the red bands are the GeneScan®-500 ROX™ internal lane standard. Clone C3 had a single transcriptional start site, as indicated by a cDNA product of 203 bases. This photo is representative of the gel images for each of the primer extension experiments.

Column-based methods for isolating total RNA tend to exclude small RNA species. Although it is beneficial to remove the tRNAs and 5S rRNAs, this size exclusion may also prevent the isolation of short mRNA species. Consequently, it may not be possible to locate the transcriptional start site of cloned DNA fragments which have the promoter adjacent to the primer binding site. Eight cDNA fragments of less than 100 bases were sized, although the majority of primer extension reactions identified promoters on larger cDNA fragments. The average size of the primer extension products was 175 bases, and most samples which failed primer extension experiments had cloned DNA inserts of approximately 100 bases

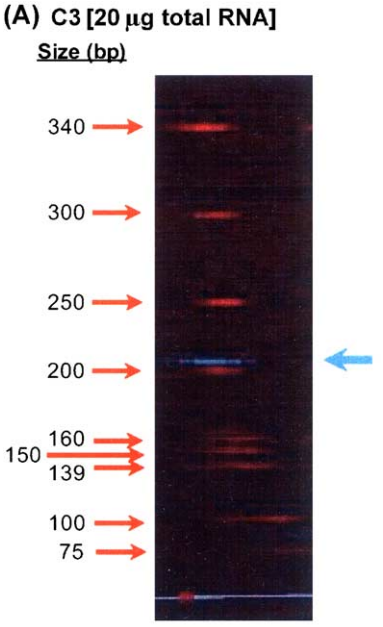

Fig. 2. Image of the gel used to analyse the primer extension product of clone C3. The blue band (blue arrow) is the FAM-labelled cDNA product. The red bands are the GeneScan®-500 ROX™ internal lane standards. The C3 primer extension product was 202.89 bases.

or less. A different method of total RNA isolation may afford greater success at sizing cDNA fragments below 100 bases. However, the FAM primer peak obscures the region around 30 bases, the smallest DNA size standard is 35 bases and the 58 base FAM signal is found in virtually all samples. Therefore, attempts to size cDNA fragments less than 60 bases may not be achievable.

3.4. Separate DNA sequencing reactions

This method of determining transcriptional start sites allows the DNA sequencing data to be analysed in conjunction with the size of the primer extension product at a later date, rather than running both samples in parallel. In this study, the DNA sequencing was completed before the primer extension experiments were conducted. The transcriptional start site(s) of the cloned promoters could then be identified using the known DNA sequence data, the location of the primer binding site on the vector and the length of the FAM-labelled cDNA primer extension product.

Fig. 3. Truncated region of clone C3 DNA sequence showing the transcriptional start site (+1), as mapped using primer extension experiments and GeneScan® analysis. The putative −10 and −35 regions with homology to the *E. coli* −10 (TATAAT) and −35 (TTGACA) consensus sequences are shown. The *H. pylori* sequence for C3 was 325 bp.

3.5. Mapped transcriptional start site for clone C3

Clone C3 contained a 325 bp fragment of *H. pylori* DNA with promoter activity. As shown in Fig. 2, a single 203 base primer extension product was obtained for C3. Fig. 3 shows the truncated sequence of clone C3 from −50 to +5, relative to the experimentally mapped transcriptional start site (+1). The putative −10 and −35 regions, with homology to the classical −10 (TATAAT) and −35 (TTGACA) *E. coli* consensus sequences, are shown.

4. Conclusions

Fluorescence-based primer extension reactions have been shown to be as sensitive as radioactive protocols (Fekete et al., 2003). Radiolabelled cDNA primer extension products can require exposure to X-ray film for up to 16 h in order to detect a signal (Fekete et al., 2003), whereas fluorescently-labelled cDNA products can be electrophoresed on an ABI 377 DNA sequencer, data analysed and the fragments sized using GeneScan® in less than 3½ h. The potential minor decrease in sensitivity (if any) has to be weighed up against the extra time, cost and effort required to conduct conventional primer extension experiments using radioisotopes.

When selecting a fluorescent marker for primer extension experiments, it is necessary to ensure there is not an overlap in the absorption or emission spectra, which could interfere with experimental results. The FAM label used in the synthesis of the PE+FAM oligonucleotide was produced by Applied Biosystems, and therefore, this fluorescent label was compatible for use with all other ABI dyes on ABI systems. Purification of primers with a 5′-FAM modification by high performance liquid chromatography (HPLC) ensures that each copy of the oligonucleotide contains a single 5′-FAM label, which corresponds to one fluorescent label per cDNA product. Therefore, the intensity of the FAM signal correlates with the amount of cDNA present in the sample, as indicated by the peak area on the electropherogram. If multiple promoters are present in a sample, the relative strength of each promoter can be inferred from the peak heights of the primer extension products. The promoter corresponding to the major transcriptional start site will produce higher levels of mRNA transcript, resulting in a stronger FAM signal. In order for GeneScan® to size primer extension products, an appropriate internal lane standard must be run with the samples. The choice of lane standard is dependent upon the fluorescent label on the primer and the GeneScan® filter set.

The ability of these primer extension reactions to be carried out in any laboratory without the need for specialised equipment is highly advantageous. Many laboratories no longer have the facilities for working with radioisotopes, whereas DNA sequencing has become a standard tool for research in molecular biology. The routine use of DNA sequencing has resulted in practically all laboratories having access to a DNA sequencing facility. The use of internal lane standards and GeneScan® software ensures the sample and standard experience the same electrophoretic forces during separation, which results in more accurate size determination.

Unlike eukaryotic mRNA, which can be isolated due to the presence of a poly(A) tail, bacterial mRNA is more difficult to isolate. This fluorescent-based primer extension protocol was shown to be more efficient using total RNA than a sample enriched for mRNA. This result was surprising, considering that a population enriched for mRNA transcripts would be expected to give more defined primer extension products. Nevertheless, when aiming to locate transcriptional start sites in bacteria, it is always preferable to work with total RNA. This fluorescent-based methodology has been optimised for use with bacterial total RNA samples.

Recently, technologies such as 5′-RACE (random amplification of cDNA ends) have been successfully used to map the transcriptional start sites of mRNA molecules. However, the 5′-RACE technique relies on the presence of a poly(A) tail at the 3′ end of the mRNA species, and therefore, this protocol is applicable only to eukaryotic cells (Schaefer, 1995; Schramm et al., 2000). A search of the literature did not reveal any adaptations of this method for use with prokaryotic organisms.

This paper describes a very useful method for identifying transcriptional start sites in a library of cloned bacterial promoters using a fluorescent-based primer extension protocol. This simple, sensitive, cost-effective approach uses total RNA and a single FAM-labelled primer to screen a large number of bacterial promoters. It requires low primer concentrations and is readily applicable to a wide range of organisms without the need for specialised equipment or facilities.

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