The Chemical Synthesis of a Gene Coding for Bovine Pancreatic DNase I and Its Cloning and Expression in *Escherichia coli*  

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A gene coding for bovine pancreatic DNase I has been constructed from synthetic oligonucleotides. This gene has been cloned into a plasmid vector pDOC55 designed to allow very tight control of expression of potentially lethal proteins. Induction of protein synthesis from the gene yielded a peptide of molecular weight of approximately 31,000, consistent with DNase I. The yield of this protein from the pDOC55 construct (pAWS) was approximately 150 μg/liter of cell culture. Attempts to clone the gene into a less tightly controlled expression vector based on the tac-promoter (pKK223-3) were unsuccessful, presumably due to the expected lethality of the product. Mutagenesis of the gene to replace the active site histidine (His-134) in the protein with glutamine yielded a gene readily clonable into both expression systems. Yields of the mutagenized protein were approximately 6 mg/liter from a pDOC55 system and 20 mg/liter from a pKK223-3 system. The activity of the proteins were assayed using the Kunitz procedure and their cleavage selectivities by digestion of the *Escherichia coli* tyr T promoter. The recombinant native enzyme had both the same specific activity and DNA cleavage selectivity as the protein isolated from bovine pancreas using these two assays. The H134Q mutant had a specific activity of about 0.001% of the native protein but had an unaltered DNA cleavage selectivity.

Bovine pancreatic DNase I (EC 3.1.21.1) is an endonuclease which cleaves double-stranded DNA to yield 5’ phosphorylated polynucleotides (Moore, 1981). DNase I does not cleave all phosphate bonds in DNA with equal ease (Lomonossoff *et al.*, 1981; Drew and Travers, 1984, 1985) and a limited digest which cleaves double-stranded DNA to yield 5'-phosphoryl oligonucleotide showed that an exposed loop of the protein centered on Y76 protruded into the minor groove of the DNA helix, the global function, i.e. a slowly changing cleavage rate, results. In contrast to the global function, neighboring phosphodiester bonds are often cut at very different rates. This effect was explained by changes in phosphate group orientation, DNase I cuts the bond between the 3’-oxygen atom and phosphorus by an S,2 general base-catalyzed nucleophilic attack of water on phosphorus (see below for details). This mechanism is most favored when water attacks the phosphorus in line with and opposite to the bond to be cleaved. Phosphate orientations allowing water access opposite to the scissile bond are easily cut, whereas other orientations are more poorly cut. As phosphate bond orientation can vary dramatically between neighboring phosphates, the local cutting function is obtained. The structure of DNase I has been solved by x-ray crystallography and this has enabled an explanation of several of the above features (Suck *et al.*, 1984, 1988; Suck and Oefner, Oefner and Suck, 1986). In particular, the 2-Å resolution structure of DNase I complexed to a short oligonucleotide showed that an exposed loop of the protein centered on Y76 protruded into the minor groove of the DNA and 2 residues (Y76 and R41) completely filled the groove. Additionally a variety of contacts were made between phosphate groups on both strands of the DNA and amino acids in the area of the exposed loop. No contacts were formed between DNase I and the major groove. The DNA was significantly distorted and was found to be bent towards the major groove with a widening of the minor groove by 3.0 Å and a consequent narrowing of the major groove. As DNase I binds in the minor groove one would clearly expect catalysis to be dependent on minor groove dimensions as suggested above. However, although poor catalysis at narrow minor groove widths (d(A-T)-rich regions) is easily explained, the low rates observed at broad minor grooves (d(G-C)-rich regions) are less easy to rationalize since the enzyme widens a “normal” minor groove of DNA when binding this molecule. It was also proposed that as DNA distortion takes place on DNase I binding, the flexibility of the minor groove might be important for catalysis. Two amino acids, E78 and H134, were implicated in catalysis. It was proposed that E78 abstracted a proton from a bound water
molecule. The resulting OH⁻ reacted as a nucleophile performing an S_n2 attack on the scissile phosphate group. This mechanism is required to explain the local cleavage effect and is also in agreement with the inversion of configuration at phosphorus observed for the DNase I-catalyzed reaction (Meid and Gerlt, 1984).

The possession of an x-ray structure for DNase I makes it an attractive target for the study of protein-nucleic acid interactions by site-directed mutagenesis. The gene for this protein has not been cloned but the amino acid sequence has been determined by conventional protein sequencing techniques (Liao et al., 1973) and found to contain 257 amino acids. The closely related pig and sheep pancreatic DNase I sequences have been investigated (Paudel and Liao, 1986a, 1986b) and were both similarly suggested to contain 257 residues. However, in light of the x-ray structure, the sequence of the bovine enzyme was modified in three respects. In particular a tripeptide IVR was inserted after the IVR sequence at positions 26, 26, and 27 giving an IVR repeat. This means that DNase I has 260 amino acids. Throughout this paper we have numbered the amino acids 1-260 to take into account the IVR insertion. In addition, T14 was changed to S14 and the positions of G and P at 227 and 228, respectively, were reversed to P and G. Rather than attempting the cloning of the gene for DNase I we have decided to synthesize it chemically based on this corrected amino acid sequence as a prelude to mutagenesis experiments. Several genes have now been assembled by chemical synthesis (Groger et al., 1988) and this method is an attractive alternative to conventional gene cloning for the following reasons. Codon usage can be optimized for the proposed organism of expression favoring high yields of the protein (Grosjean and Fiers, 1982). Restriction sites can be built into the gene (using the redundancy of the genetic code) greatly facilitating subsequent gene manipulation and allowing mutagenesis by the cassette method. Specifically for pancreatic DNase I it is anticipated that it would be difficult to obtain the mRNA coding for the protein (for cDNA preparation) as the pancreas is known to be particularly rich in RNases. This paper describes the synthesis of a gene which codes for bovine pancreatic DNase I based on the amino acid sequence described above and also for several variants. In particular we show that the corrected amino acid sequence is still wrong. The most important mistake being that residues E38 and Q39 should be reversed to Q38, E39. Three variants have been prepared; the first based on the published amino acid sequence (E38, Q39), the second having these residues reversed (Q38, E39), and the third having the active site histidine (H134) replaced by glutamine (Q38, E39, H134Q). We show that the first and third variants have very low DNase I activity and the genes can be overexpressed in *E. coli* in plasmid pKK223-3 (Fig. 1a) under the control of the tac promoter (Brosius and Holy, 1984). The second construct gives an active DNase I but the gene is too lethal to be expressed in *E. coli* using this plasmid. However, small quantities of active DNase I can be produced in *E. coli* using the plasmid pDOC55 (Fig. 1b) designed for very tight control of protein expression (O'Connor and Timmis, 1987). Based on studies with partially purified active DNase I we show that the recombinant protein has the same specific activity and the same cleavage selectivity for the *E. coli* tyr T promoter as the enzyme isolated from bovine pancreas.

**EXPERIMENTAL PROCEDURES**

All restriction endonucleases and DNA modifying enzymes were obtained from Boehringer Mannheim (Lewes, United Kingdom), New Hampshire Biologicals Ltd. (Cramlington, United Kingdom), or New England Biolabs (Beverley, MA) and were used according to manufacturer's protocols. Chemicals were obtained from BDH Ltd. (Poole, United Kingdom) and chromatography materials were obtained from Pharmacia LKB (Uppsala, Sweden). E. coli strains used were: -TG1 (Carter et al., 1986), -lac-proAB, supE, thi, bsd55, F', traD36, proAB, lacI, lacZAM15), JM103 (Yanisch-Perron et al., 1985, Δ(lac-proAB), supE, thi, rpsL, endA, sbcB15, F', traD36, proAB, lacI, lacZAM15), JM83 (λ-Vierra and Mesung, 1982, F', ara, Δ(lac-proAB), thi, rpsL, 860, Δ(lacZM15), λ) M72 (Bernard et al., 1979; Δ(bio-uvr), lacZAm, rpsL, Δ(trp E2) (λ, Nam7, Nam53, cl587, ΔH1)), M13mp19 (Boehringer Mannheim), and pKMΔ-98 (gift from Dr. K. R. Fox, Southampton University). Bacteria containing plasmids were grown in LB broth (Maniatis et al., 1982) containing ampicillin (50 µg/ml) at the temperature given in the text. All standard DNA manipulations and electrophoresis procedures were carried out as previously described (Maniatis et al., 1982) unless otherwise stated in the text. Proteins were analyzed by SDS-polyacrylamide electrophoresis (Laemmli, 1970) and amino acid sequence analysis was performed using Applied Biosystems 477A pulsed liquid phase protein sequencer. On-line phenylthiohydantoin-derivative identification was made using an Applied Biosystems 120A analyzer. Protein concentrations were determined approximately by measuring the absorbance of protein solutions at 290 nm (a 1 mg/ml solution of DNase I has an A280 of 1.25).  

**Gene Design**

The gene was designed (Fig. 2) with the help of commercially available software (Unger, 1986).

**Oligonucleotides**

Oligonucleotides were synthesized on a 0.2-µm scale on an Applied Biosystems 381A DNA synthesizer using standard phosphoramidite chemistry. They were purified by electrophoresis on 20% polyacrylamide gels containing 8% urea and visualized by UV shadowing. The products were eluted from the gel by incubation at 37 °C in 100 mM Tris, 0.5 M NaCl, 5 mM EDTA, pH 8.0. 2-3 nmol of oligonucleotide were 5'-phosphorylated using T4 polynucleotide kinase (18 units). Oligonucleotides were diluted to a final concentration of 10 pmol/µl (concentrations were determined by measuring the absorbance of solutions at 260 nm). Oligonucleotides 1A and 1B carry the self-complimentary 5'-overlaps of the complete gene and were therefore not 5'-phosphorylated to prevent concatemer formation on treatment with T4-DNA ligase.

**Ligation**

Oligonucleotide ligations to give the four gene quarters were carried out on a 200-pmol scale in a final volume of 200 µl. After annealing oligonucleotide pairs by a heating/slow cooling cycle, incubation was at 37 °C for 5 h with T4 DNA ligase (40 units). The resultant products were purified by electrophoresis on a 20% polyacrylamide gel run under nondenaturing conditions. Bands corresponding to products of the correct molecular weight (equivalent to approximately 200 base pairs) were visualized by UV shadowing and eluted from the gel as described above. Final ligation of the four gene quarters to give the required 816-base pair fragment was carried out by mixing the products described above with T4 DNA ligase (2 units) in a total volume of 70 µl and incubating at 37 °C for 1 h. This gene was used for cloning into M13mp19 without further purification.

1 The abbreviations used are: SDS, sodium dodecyl sulfate; IPTG, isopropyl-β-D-thiogalactoside.
2 D. Suck, personal communication.
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Cloning of the Gene into M13 and Sequencing

The DNase I gene prepared above was ligated into M13mp19 (Yanisch-Perron et al., 1985) previously cut with EcoRI and HindIII. Thus 0.1 μg of the linear M13mp19 was ligated with varying amounts (Yanisch-Perron et al., 1985) previously cut with EcoRI and HindIII in an attempt to reisolate the 816-base pair HindIII fragment from the appropriate recombinant M13mp19 rep-2

Subcloning into pDOC55

pDOC55 was cleaved with Smal and HindIII, and dephosphorylated as above. Double-stranded DNA was ligated with synthetic oligonucleotides; boxed areas indicate the recognition sites of the restriction sites in the gene; ****+, indicates the Shine/Dalgarno site from the A protein of phage R17 (see text for details).

FIG. 2. The design for the synthetic gene coding for DNase I (Q38E, E38Q). Lines delineate the synthetic oligonucleotides; boxed areas indicate the recognition sites of the restriction sites in the gene; ****+, indicates the Shine/Dalgarno site from the A protein of phage R17 (see text for details).

Optimal Time

DNase I Induction

pKK223-3 Derivatives—E. coli JM103[pAW2] were grown to an A360 of approximately 0.4 at 37 °C. IPTG was added to a concentration of 0.2 mM and aliquots of cells were removed at time intervals. The cells were pelleted by centrifugation and either total or soluble fractions were analyzed. For total protein the cells were resuspended in 62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.05% bromphenol blue, and boiled for 15 min. These proteins were analyzed on a 12.5% polyacrylamide SDS gel and were visualized by Coomassie Blue stain. Gels were scanned using a Chromoscan 3 gel scanner (Joyce-Loebl, United Kingdom). For soluble protein, cells were suspended in 10 mM Tris, 2 mM CaCl2, 100 μM benzamidine, 100 μM phenylmethylsulfonylfluoride, pH 7.6, and sonicated at 4 °C.
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**Results**

Cloning of the 816-base pair synthetic DNA fragment into M13mp19 yielded a 160-base pair fragment. The fragment was 
edenatured by heat treatment to yield a 500-base pair fragment in disagreement with the published sequence. Digestion with 
SmaI and HindIII sites in this vector. When recombinant pDOC55 clones are plated on LB agar containing IPTG and 
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, the colonies obtained are blue, despite the insertion of the DNase I 
gene into the lacZ gene vector. These problems associated with the use of pDOC55 are the subject of continued 
studies by the original authors. The subcloning of the gene from M13mp19DNaseIrec5 yielded pAW2. After heat induc-
tion of M72 cells carrying this construct no new protein at the expected molecular weight could be observed on a 
Coomassie Blue-stained polyacrylamide gel (Fig. 4). However, DNase I activity was easily detectable in the cleared cell 
solucates prepared as described above using the Kunitz assay. This activity was absent from similarly induced 
M72[pDOC55] or M72[pAW6] cells (pAW6 carries a gene

| TABLE I | Details of plasmids and M13 clones constructed |
|---------|-----------------------------------------------|
| Plasmid/M13 |
| M13mp19DNaseIrec2 | TG1 | Q38E, E39Q | M13mp19 |
| M13mp19DNaseIrec5 | TG1 | DNase I | M13mp19 |
| pAW2 | JM103 | Q38E, E39Q | pKK223-3 |
| pAW4 | JM104 | H134Q | pKK223-3 |
| pAW5 | M72/JM83 (A) | DNase I | pDOC55 |
| pAW6 | M72/JM83 (A) | H134Q | pDOC55 |

**Protein Purification**

Cells isolated from large scale inductions were frozen and stored at -20 °C. Four g of induced cells were thawed and resuspended thoroughly in 40 ml of 10 mM Tris, 2 mM CaCl₂, 100 μM benzamidine, 
100 μM phenylmethylsulfonyl fluoride, pH 7.6. The cells were disrupted by sonication at 4 °C. The insoluble debris was removed by centrifugation at 40,000 x g, 4 °C, for 1 h. The soluble proteins were 
applied to a column of DEAE-Sephacel (10 x 3 cm²) equilibrated to and eluted with the above buffer and the unbound proteins were washed through with this buffer. Bound proteins were eluted with a gradient of 0-0.3 M 
NaCl in the same buffer over 1 liter, run at 60 ml/h. Proteins were detected by absorbance at 280 nm or by activity in the Kunitz assay. The desired fractions were concentrated to 2 ml using Centriprep 10 spin concentrators (Amicon). The resultant solution was applied to a Sephadex G75-SF column (90 x 3 cm) equilibrated to and eluted with 10 mM Tris, 2 mM CaCl₂, 30 mM NaCl, pH 7.6, at 20 ml/h. The eluate was analyzed as before and by SDS-polyacrylamide gel electrophoresis. The fractions containing the purest protein were combined and concentrated. Purified protein was shock frozen in liquid nitrogen and stored at -70 °C.

**Kunitz Assay**

The Kunitz assay for DNase I activity (Kunitz, 1950) was performed at pH 8.0 in 10 mM Tris buffer in the presence of 0.1 mM 
CaCl₂ and 1 mM MgCl₂ using 0.05 mg/ml calf thymus DNA (Sigma) as substrate. 50 ml of 10 mM Tris, 2 mM CaCl₂, 100 μM benzamidine, 
100 μM phenylmethylsulfonyl fluoride, pH 7.6. The cells were disrupted by sonication at 4 °C. The insoluble debris was removed by centrifugation at 40,000 x g, 4 °C, for 1 h. The soluble proteins were 
applied to a column of DEAE-Sephacel (10 x 3 cm²) equilibrated to and eluted with the above buffer and the unbound proteins were washed through with this buffer. Bound proteins were eluted with a gradient of 0-0.3 M 
NaCl in the same buffer over 1 liter, run at 60 ml/h. Proteins were detected by absorbance at 280 nm or by activity in the Kunitz assay. The desired fractions were concentrated to 2 ml using Centriprep 10 spin concentrators (Amicon). The resultant solution was applied to a Sephadex G75-SF column (90 x 3 cm) equilibrated to and eluted with 10 mM Tris, 2 mM CaCl₂, 30 mM NaCl, pH 7.6, at 20 ml/h. The eluate was analyzed as before and by SDS-polyacrylamide gel electrophoresis. The fractions containing the purest protein were combined and concentrated. Purified protein was shock frozen in liquid nitrogen and stored at -70 °C.

**Tyr T Promoter DNA Cleavage**

Plasmid pKM101 (Drew and Travers, 1984) was cleaved with EcoRI and AatII to yield a 160-base pair fragment. The fragment was treated with [α-32P]ATP (Amersham Int. United Kingdom, 3000 Ci/ 
mmol) and reverse transcriptase to label the 3'-5' single-stranded end (the EcoRI cleaved end). Digestions of the DNA were carried out essentially as described before (Drew and Travers, 1984) in the presence of 0.5 mM MgCl₂, 0.5 mM MnSO₄, 10 mM NaCl, 5 mM Tris, 
pH 8.0. Aliquots of the digests were removed at 1, 5, and 30 min and the reaction stopped by adding the samples to an equivalent volume of 0.1% bromphenol blue in formamide. Samples were 
boiled immediately for 10 min and separated by electrophoresis on an 8% polyacrylamide gel in the presence of 7 M urea. Autoradiography was performed at -70 °C with an intensifying screen for 16 h.

**Results**

Cloning of the 816-base pair synthetic DNA fragment into M13mp19 yielded a single recombinant phage from which the fragment could be isolated. Sequencing of the gene revealed a single deletion of an T-A base pair at position 609. This was correct by cassette mutagenesis using the Sall and SpeI restriction sites. Subcloning of this gene from using a Soniprep 100 sonicator (M. S. E., United Kingdom). The sonicate was centrifuged to precipitate insoluble material and the soluble proteins were analyzed by SDS-gel electrophoresis as above.

**pDOC55 Derivatives—E. coli M13/pAW6** were grown to an A₅₀₀ of approximately 0.4 at 30 °C in the presence of 2 mM IPTG. An equivalent volume of LB broth at 54 °C was added to immediately increase the culture temperature to 42 °C. Aliquots of cells were taken and pelleted by centrifugation. Soluble proteins prepared as above were monitored for DNase I activity using the Kunitz assay and this activity was expressed as units per ml of cell culture.

**protein activity was expressed as units per ml of cell culture.**
subcloned from M13mp19DNaseIrec5 which directs the synthesis of DNase I (H134Q), H-134 being the active site histidine required for enzyme activity. This clearly ascribes the observed nuclease activity to a recombinant DNase I. The optimum time of induction for the production of recombinant DNase I is 2-5 h (Fig. 6). This protein was produced at levels from 0.1 to 1 mg/liter of cell culture and was partially purified (Fig. 5) using an identical two-column procedure to that used for DNase I (Q38E, E39Q). The specific activity of this protein corrected for purity was found to be identical to that of the native protein, measured at 5 x 10^9 units/g of protein. Similarly, DNase I (H134Q) was purified from induced JM103[pAW4] and M72[pAW6]. The M13mp19 clone which carries the DNase I (H134Q) gene was prepared from M13mp19DNaseIrec5 using the XhoI and BssHII sites. The protein was easily purified from JM103[pAW4] (approximately 20 mg/liter of cell culture) and partially purified (Fig. 5) from M72[pAW6] (approximately 6 mg/liter of cell culture, estimated by gel scanning to determine protein purity). As expected, DNase I (H134Q) is inactive in the Kunitz assay.

The cleavage selectivities of the three recombinant proteins towards the tyr T promoter DNA have been examined. Recombinant DNase I and native enzyme have identical hydrolysis patterns (Fig. 7). Despite the apparent inactivity of DNase I (Q38E, E39Q) and DNase I (H134Q) in the Kunitz assay, they show activity (when present in large amounts) in this more sensitive assay. The relative activities of the two enzymes can be estimated at approximately 0.01 and 0.001% of the native activity. While DNase I (H134Q) shows an identical hydrolysis pattern to native enzyme, that for DNase I (Q38E, E39Q) is somewhat altered.

**DISCUSSION**

A gene coding for bovine pancreatic DNase I, based on the currently published (Liao et al., 1973; Ofner and Suck, 1986) amino acid sequence, has been successfully constructed by the annealing and ligation of 32 synthetic oligonucleotides (Fig. 2). The gene was designed with the following features in mind. (a) Unique restriction sites were placed throughout the gene to facilitate mutagenesis by the cassette method. (b) As far as possible the codon usage is that found in highly expressed genes in E. coli (Groisman and Fiers, 1982). (c) A Shine-Dalgarno sequence of 7 bases (AGGAGGT) was placed at nucleotides -8 to -14 with respect to the methionine start codon. This sequence was taken from the mRNA coding for the A protein of R17 phage which is highly expressed protein (Shine and Dalgarno, 1974; Steitz, 1969). (d) Two stop codons (TAA, TAG) were placed after the final amino acid codon to ensure efficient termination of translation with minimal read through. (e) The total 816-base pair fragment carries EcoRI and HindIII cohesive ends. Each of the synthetic oligonucleotides is about 50 bases in length. We have investigated overlaps of 4, 5, and 7 base pairs for the DNA ligase catalyzed joining of synthetic duplexes. Many investigators have used short 4-base overlaps for gene assembly presumably to minimize the chances of incorrect ligations. We observed much higher ligation yields with the 7-base pair overlaps and would strongly recommend this number as the minimal overlap. Attempts to ligate all 32 oligonucleotides in one pot and therefore assemble the gene in a single step were unsuccessful. Therefore we adopted a strategy of an initial ligation of four quarters of the gene.

The differences in amino acid sequence between our initial protein product and the native bovine enzyme are shown in Table II. We do not believe that the NH2-terminal methionine, serine/threonine-14 differences, or lack of glycosylation at position -18 are responsible for the lack of enzymic activity of this protein, these changes are all at the surface of the protein and would not be expected to be involved in substrate recognition or catalysis. We believe that the key difference lies at positions 38 and 39. The original sequence was Q38, Q39, however, our data shows that the true sequence in the bovine enzyme is Q38, E39. Interestingly a complete sequence of the pig enzyme also showed QE at this position (Paudel and Liao, 1986a). With the sheep enzyme this sequence was assigned as EQ (Paudel and Liao, 1986b). However, the methods used were not based on complete sequencing but rather peptide isolation, amino acid composition determination, and sequence assignment by homology comparison with the bovine enzyme. An incorrect EQ assignment with the bovine protein would automatically lead to this mistake for the sheep enzyme. Examination of the crystal structure of DNase I shows that the amino acid at position -39 has an important catalytic role, coordinating the essential metal ion (Ca2+ or Mg2+/Mn2+ in vivo). Obviously glutamine would not be able to bind a metal ion as efficiently as glutamate. Due to the inherent errors in protein sequencing and also crystallographic difficulties in distinguishing acid and amide amino acids, we cannot be sure if the sequence we have prepared represents the true DNase I sequence. Nevertheless the identical specific activities and tyr T promoter cleavage selectivities seen between recombinant DNase I and the bovine enzyme (see below) mean that any amino acid differences are silent mutations with no effect on DNA binding and catalysis. We are currently about 70% of the way through a complete resequencing of the bovine enzyme and will report our results more fully elsewhere.

We ascribe the lack of success in subcloning the DNase I

**TABLE II**

**Results from protein sequencing**

| Sequence | 1 | 10 | 20 | 30 | 40 |
|----------|---|----|----|----|----|
| Bovine enzyme | LKIAAFNRTHFGETKMSXATLASYIVRIVRYDVILFEVQVRS5HL | | | | |
| DNase I (Q38E, E39Q) | MLKIAAFNRTHFGEKMSXATLASYIVRIVRYDVILFEVQVRS5HL | | | | |
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FIG. 4. SDS-polyacrylamide gel of total cellular proteins from cells after induction as described in the text. a, JM103[pAW4]; b, JM103[pKK223-3]; c, M72[pAW5]; d, M72[pAW6]; e, M72[pDOC55]. Arrow marks the expected position of recombinant DNase I. Molecular masses of standard proteins are given in kilodaltons.

FIG. 5. SDS-polyacrylamide gel of purified DNase I derived from (a) JM103[pAW2] (DNase I (Q38E, E39Q)), (b) M72[pAW5] (recombinant DNase I), (c) M72[pAW6] (DNase I (H134Q)). Lane d is purified bovine pancreatic DNase I. Molecular masses of standard proteins are given in kilodaltons. Purity of proteins as determined by gel scanning (see text) are: a, > 95%; b, 12%; c, 60%.

FIG. 6. Graph showing time course of induction of recombinant DNase I from M72[pAW5].

gene from M13mp19DNaseIrec5 into pKK223-3 to the toxicity of the gene product and the lack of total control over protein synthesis provided by the lac-promoter. This was overcome by the use of pDOC55. This vector has been used to clone EcoRI endonuclease in the absence of the EcoRI methylase (O'Connor and Timmis, 1987). Expression of cloned genes is under the control of the λpL promoter which is highly repressed in E. coli hosts that produce the λ-repressor either as the wild type (JM83(λ)) or as a temperature-sensitive mutant (M72). However, expression of proteins is even further reduced in the repressed state by the presence of an antisense lac-promoter downstream of the cloned gene. Thus if the cells are grown in the presence of a lac-inducer, the lac-promoter directs the synthesis of mRNA, antisense to the normal sense message derived from the gene. It is assumed that if any sense message is produced from the λpL-promoter, this will form an RNA-duplex with the antisense message which is incapable of being translated by the ribosome. We have been unable to clone the DNase I gene under the control of the λpL-promoter alone,\(^3\) showing that in M72 cells this promoter is not sufficiently tightly controlled and the presence of the antisense promoter is an absolute requirement. Yields of the inactive mutants from the pDOC55 expression system were always a little lower than those obtained from the pKK223-3 system, perhaps indicating a slight repression of synthesis caused by the antisense lac-promoter. However, the comparatively small and variable amount of active DNase I produced by the pDOC55 system is clearly a function of the toxicity of this gene product in E. coli. We have tried to utilize the secretion system offered by the plasmid pIN-III-ompA (Ghrayeb et al., 1984) as a possible method for overcoming the toxicity of DNase I, but with no apparent success.

Although we have yet to purify the recombinant active DNase I to homogeneity, it is quite clear that the nuclease activity that we have partially purified is authentic DNase I. First, nuclease activity is easily detected in lysed cell suspensions of M72[pAW5] after induction by heat, but not of M72[pAW6] nor of M72[pDOC55]. Second, the partially purified nuclease has the same specific activity as bovine pancreatic DNase I when corrected for purity. However, the best evidence comes from the pattern of hydrolysis produced by DNase I on the tyr T promoter DNA from E. coli. Authentic bovine pancreatic DNase I gives a characteristic partial cleavage pattern (Fig. 7) and this pattern has been shown to be different for other nucleases such as DNase II (Drew and Travers, 1984), micrococcal nuclease (Fox and Waring, 1987), and S1 nuclease (Drew et al., 1985). Protein derived from M72[pAW5] gives an identical cleavage pattern to bovine

\(^3\) A. Doherty, unpublished observations.
DNase I and therefore shows the same characteristic selectivity in its cleavage reaction. It would be extremely unlikely that a nuclease contamination from E. coli would show an identical cleavage selectivity to the bovine protein. Fig. 7 also shows that at high concentrations (approximately $1.5 \times 10^8$ times that for active recombinant DNase I) DNase I (H134Q) shows DNA cleavage and furthermore, the cutting selectivity is identical to the bovine protein. Given the role of H134 in the mechanism of DNase I as an essential base that serves to activate water during the hydrolysis reaction (Suck and Oefner, 1986) it is perhaps surprising that DNase I (H134Q) shows any activity at all. A possible rationale is that although this enzyme lacks its catalytic base it behaves in a similar manner to a catalytic antibody. One would expect the DNase I (H134Q) to bind preferentially to the transition state of the substrate DNA and therefore to stabilize this state, possibly leading to a low catalytic rate. The identical cleavage selectivity shown by DNase I (H134Q) as compared to the bovine enzyme is not surprising as the X-ray structure shows that H134 plays no role in DNA binding. However, at this early stage it is not possible to deduce the quantitative importance of H134 in the catalytic efficiency of the enzyme. In another similar case (Hibler et al., 1987) the removal of the general base catalyst Glu-43 from staphylococcal nuclease and replacement with either charged or uncharged amino acids led to global changes in protein structure, making interpretation of kinetic effects difficult. We have also shown that DNase I (Q38E, E39Q) is also active when assayed by tyr T cleavage. Interestingly, this variant shows some changes in the selectivity of DNA cleavage when compared with the other proteins studied. The global cleavage pattern is not affected although we see several significant changes in some of the local cleavage effects. As amino acid -39 binds the essential metal ion, these local changes could be due to a repositioning of this cation changing the susceptibility of individual bonds to hydrolysis. These effects will be reported in more detail in a later publication.

Our research is currently taking two directions. First, we are trying to engineer the production of active DNase I recombinants in larger quantities. Second, we are producing and characterizing interesting mutants of DNase I based on the crystal structure. These efforts will be reported on as and when they succeed.

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