Cloning and expression of human salivary-gland kallikrein in *Escherichia coli*

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CDNA clones for human kallikrein have been identified in a cDNA library constructed from mRNA of human salivary gland. The entire coding sequence for preprokallikrein and for the 5'- and 3'-untranslated regions were isolated by using a mixture of oligonucleotides corresponding to amino acids 51–56 of human urinary kallikrein and one oligonucleotide corresponding to amino acids 233–238 of human pancreatic kallikrein. The DNA sequence proved that, with the exception of two amino acid exchanges, kallikrein of the human salivary gland is identical with pancreatic kallikrein. Salivary gland and renal kallikrein was expressed in *Escherichia coli* from plasmid pKK223-3 under the control of the *tac* promoter. The protein was identified by Western-blot analysis and by demonstration of its specific proteolytic activity.

INTRODUCTION

Tissue kallikreins are serine proteinases of closely related structure. They are characterized by their affinity to the low- and high-molecular-mass kininogens [1]. From these primary physiological substrates tissue kallikrein liberates the peptide kinin (Lys-bradykinin or kallidin) by cleavage of specific peptide bonds [2]. Kinin produces vasodilatation, triggers smooth-muscle contraction of uterus, bronchi and ileum [3] and stimulates cellular glucose uptake [4] and sperm motility [5]. Until now kallikrein has been found in a variety of tissues such as pancreas [6], parotid and submandibular glands [7], kidney, testes and intestine [6]. Recently kallikrein could also be identified in some other tissues hitherto considered to be devoid of this class of proteinases. These tissues include spleen [8], brain [9] and vascular wall [10]. Tissue kallikreins are glycoproteins with molecular masses between 25 and 40 kDa because of the heterogeneity in their carbohydrate content [2]. They have been purified and characterized from various human tissues (for a review, see [11]). For human pancreatic kallikrein the complete amino acid sequence of preprokallikrein was determined by cloning and sequencing its corresponding cDNA [12]. Comparison of 31 N-terminal residues of pancreatic kallikrein with available data of kallikrein from urine [13] revealed complete identity [12].

Over the past few years the concept of a kallikrein multigene family has been generally accepted. This concept is not simply based on the specific enzymic function of the gene products, but essentially on sequence identity. The members of this family include not only the gene for kallikrein, with its activity in kallidin production, but also genes coding for proteinases of similar amino acid sequence such as enzymes that process nerve-growth-factor precursor [14] and epidermal-growth-factor precursor [15]. Among different mammals the numbers of

genes encoding kallikrein and related proteins vary greatly. In mouse the kallikrein gene family consists of 24 genes of which at least ten are expressed and several appear to be pseudogenes [16]. The number of members of this gene family may vary between 17 in the rat [17] and three in the hamster [18]. The human kallikrein gene family contains at least four distinct genes: the kallikrein gene, an unidentified partial gene sequence [12,19], a characterized, but unidentified, gene termed 'hGk-1' [20] and the gene coding for prostate-specific antigen [21,22]. A major prerequisite for understanding of the molecular organization and the structural and perhaps functional differences among members of the kallikrein gene family is the availability of kallikrein in homogeneous form. As a first step towards this goal we report the isolation of the complete cDNA coding for human preprokallikrein from salivary gland and its expression in *Escherichia coli*.

MATERIALS AND METHODS

Materials

Enzymes were from Boehringer Mannheim (Mannheim, Germany) or Bethesda Research Laboratories (Eggenstein, Germany), radiolabelled nucleotides were from Amersham (Braunschweig, Germany) and goat anti-rabbit IgG-alkaline phosphatase conjugate was purchased from Sigma (München, Germany). Human salivary glands were obtained as autopsy material and stored in liquid N₂.

Methods

Total RNA and polyadenylated RNA were isolated as described in [23]. Single-stranded and double-stranded cDNA were synthesized by the method of Gubler & Hoffman [24] using oligo(dT)-primed reverse transcription of poly(A)-rich RNA (reverse transcriptase; W,

Abbreviations used: IPTG, isopropyl β -D-thiogalactopyranoside; PAGE, polyacrylamide-gel electrophoresis; the compositions of NET, Luria-Bertani medium and STE are given in the text.

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These sequence data have been submitted to the EMBL/GenBank/DDJ Nucleotide Sequence Databases under the accession number X13561.

Beard, Life Sciences, St. Petersburg, FL, U.S.A.). The dC-extended cDNA was inserted in restriction-*endo*-nuclease-*Pst*I-hydrolysed, dG-tailed pUC9 (Pharmacia) and transformed into *E. coli* DH2 by using the method of Hanahan [25]. The cDNA library was transferred to Hybond N (Amersham International) and prepared for colony hybridization as described by Thayer [26].

Oligonucleotide synthesis and hybridization. Oligonucleotides and sequencing primers were synthesized on a DNA Synthesizer Type 380 A from Applied Biosystems, purified by reversed-phase h.p.l.c. and analysed by phosphorylation and subsequent analytical gel electrophoresis in denaturing polyacrylamide gels [27]. Hybridization with oligonucleotides was done as described by Wallace *et al.* [28]. For detection of kallikrein-specific mRNA sequences the filters were prehybridized in $6 \times \text{NET}$ [$20 \times \text{NET}$ is $3 \text{ M-NaCl}/0.3 \text{ M-Tris}/\text{HCl}$ (pH 8.3)/ 20 mM-EDTA], $10 \times \text{Denhardt's}$ [29], 0.1% (w/v) SDS and $100 \mu\text{g}$ of tRNA/ml at 65°C for 3 h. Hybridization was performed overnight in the same solution with the exception that tRNA was substituted by ^{32}P -labelled oligonucleotide [27] with $(1-2) \times 10^6$ c.p.m./ml. Hybridization temperature for oligonucleotide AR2 was 39°C and for KK2 50°C . Colony filters were prehybridized in $6 \times \text{NET}$, 0.5% (w/v) Nonidet P40 and $100 \mu\text{g}$ of tRNA/ml and hybridized in prehybridization solution with ^{32}P -labelled oligonucleotides at $(1-2) \times 10^6$ c.p.m./ml instead of tRNA under the same conditions as those described above. Hybridized filters were washed once in $0.9 \text{ M-NaCl}/0.09 \text{ M-sodium citrate}$, pH 7.2, at 4°C for 5 min, twice at room temperature, twice at 40°C for 5 min and finally once at 50°C for 5 min. Filters were blotted dry and exposed to Kodak XAR X-ray film between two intensifier screens (Cronex Lightning Plus).

DNA sequence analysis. Plasmid DNA was prepared by the method of Hardies *et al.* [30]. The cDNA fragment encoding kallikrein was sequenced by a combination of the plasmid dideoxy chain-termination method [31] and by the method of Maxam & Gilbert [32]. Regions of the cDNA showing differences from published human kallikrein coding sequences [12,19,20,33] were sequenced three times.

Expression of kallikrein in *E. coli*. *E. coli* BMH 71/18 containing either recombinant plasmid pKA21 or pSP6 [34] were grown to $0.8 A_{578}$ unit/ml at 37°C in Luria-Bertani medium (per litre: 10 g of Bacto tryptone, 5 g of Bacto yeast extract and 10 g of NaCl) containing 50 mg of ampicillin/l. Expression of kallikrein under the control of the *tac* promoter was induced by adding isopropyl β -D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. At different times after induction the amount of cells corresponding to $2 A_{578}$ units was centrifuged for 1 min and resuspended in $200 \mu\text{l}$ of STE [25% (w/v) sucrose/ $50 \text{ mM-Tris}/\text{HCl}$ (pH 7.2)/ 10 mM-EDTA]. After 15 min incubation at 0°C and subsequent centrifugation for 5 min at $8000 g$, cells were resuspended in $200 \mu\text{l}$ of prechilled water and incubated for a further 30 min at 0°C . Periplasmic proteins were separated from cell debris by a 10 min centrifugation at $12000 g$. For immunological detection of kallikrein, $200 \mu\text{l}$ of the periplasmic fraction ($2 A_{578}$ units) were subjected to SDS/ 12.5% -(w/v)-polyacrylamide-gel electrophoresis

(PAGE) [35] and transferred to nitrocellulose filter as described by Towbin *et al.* [36]. The filters were incubated overnight at 4°C with the antiserum against kallikrein, followed by another 2 h incubation with the second antibody at room temperature. Bands were revealed by addition of 4-chloronaphthol and H_2O_2 . Proteolytic activity of kallikrein was measured by the method of Fiedler *et al.* [37].

RESULTS AND DISCUSSION

Isolation and sequence determination of human salivary-gland kallikrein cDNA

From preliminary amino-acid-sequence data of human urinary kallikrein (R. Geiger, unpublished work) and from published data [13] of human pancreatic kallikrein, two oligonucleotides were synthesized. One oligonucleotide encoded the amino acids 51–56 (probe AR 2) of urinary kallikrein. Considering the third-base degeneracies in amino acid codons a 17-mer oligonucleotide mixed probe was constructed which contained all possible DNA sequences. This probe was only used to probe mRNA isolated from salivary-gland tissue for the presence of kallikrein-encoding molecules. The second oligonucleotide (probe KK2) was deduced from the cDNA sequence for pancreatic kallikrein [12], encoding the C-terminal amino acids 233–238. This probe proved useful for Northern-blot analysis as well as for the isolation of recombinant kallikrein-coding plasmids.

mRNA was prepared from salivary gland and screened by Northern blot analysis with both oligonucleotides as hybridization probes (Fig. 1). In both cases a single

Fig. 1. Northern-blot analysis of salivary-gland RNA

(a) A portion ($10 \mu\text{g}$) of poly(A) mRNA (lane 1) and $10 \mu\text{g}$ of total RNA from human salivary gland (lane 2) were size-fractionated on a glyoxal-agarose gel and transferred to Hybond N membrane. The filter was hybridized with ^{32}P -labelled mixed oligonucleotide AR2. (b) Portions ($10 \mu\text{g}$) of poly(A) mRNA from two different RNA preparations were applied in slot 1 and 2. Hybridization was carried out with ^{32}P -labelled oligonucleotide KK2, being complementary to 3'-sequence of human pancreatic kallikrein mRNA. (c) A portion ($10 \mu\text{g}$) of poly(A) mRNA from human salivary gland (lane 1) and $5 \mu\text{g}$ of human pancreas (lane 2) were size-fractionated and subsequently analysed by Northern-blot hybridization with human pancreas kallikrein-specific oligonucleotide KK2 as hybridization probe.

Fig. 2. Nucleotide and protein sequence of human salivary gland kallikrein

(a) The *N*-terminal amino acid (isoleucine) of mature kallikrein is labelled as amino acid +1. Positions of the oligonucleotides AR2 and KK2, as well as the presumed polyadenylation signal, are underlined. (b) Sequencing strategy and primary structure of human salivary-gland kallikrein cDNA. Each arrow indicates the extent and direction of one sequencing reaction.

Table 1. Differences in the amino acid and DNA sequences between pancreatic [12], renal [19] and salivary-gland kallikrein described here and the kalikrein gene [33].

Amino acid position	Salivary-gland kallikrein cDNA	Pancreas kallikrein cDNA	Renal kallikrein cDNA	Kallikrein gene
Residue 111	Asp	Asp	Asp	Asp
Codon	GAT	GAC	GAC	GAC
Residue 121	Glu	Gln	Gln	Gln
Codon	GAG	CAG	CAG	CAG
Residue 162	Lys	Glu	Glu	Lys
Codon	AAA	GAA	GAA	GAA

strong hybridization signal was obtained. In order to correlate the length of the mRNA with that reported for human pancreatic kallikrein, an additional Northern-blot analysis was performed with poly(A)⁺ RNA isolated from human pancreas. As previously observed [12], the mRNA detected in both tissues had the same size, namely of about 1000–1100 nucleotides in length (Fig. 1c). The mRNA isolated from salivary gland was subsequently used to synthesize double-stranded cDNA as described by Gubler & Hoffman [24]. The cDNA was fractionated according to size, and material longer than 600 bp was extended with d(C) residues, annealed to d(G)-tailed *Pst*I-cleaved pUC9 DNA and used to transform *E. coli* DH2 as described previously [25].

A cDNA library of about 3500 transformants was obtained and screened with probe KK2. Out of ten colonies which gave significantly stronger hybridization signals than background, one recombinant plasmid with the largest cDNA insert was chosen and further analysed by Southern-blot hybridization. The *Pst*I insert of this recombinant plasmid was 940 bp in length, excluding the extended dG/dC tails. The cDNA sequence obtained is shown in Fig. 2. It contains an open reading frame, beginning with an ATG codon at positions 37–39 and ending with an TGA translation termination codon at position 824–826. The open reading frame of this clone codes for 262 amino acids and shows nearly complete identity with the gene for the human pancreatic kallikrein both on the DNA and amino-acid-sequence levels. In the 3'-untranslated region a variant sequence AGTAAA of the canonical polyadenylation site [38,39] was found. This sequence presumably serves as polyadenylation signal, because no other sequence could be found fitting with the consensus sequence. The 5'-untranslated region consists of 36 nucleotides and corresponds exactly in length and nucleotide sequence to that reported for pancreatic kallikrein [12]. Comparison of the cDNA sequences of human salivary-gland, pancreatic and kidney [19] kallikrein and the kallikrein gene [33] revealed complete identity, with the exceptions of two amino acid exchanges in position 121 and 162 caused by a single nucleotide exchange and one 'silent' mutation in the triplet for amino acid 111 (Table 1). The high degree of sequence identity between the salivary-gland kallikrein cDNA report here and the pancreatic [12] and renal kallikrein cDNA [19,33] confirms the previous suggestion that the kallikreins expressed in these various tissues are all encoded by a single gene [12]. The observed amino acid substitutions (amino acids 121 and 162), as well as the silent mutation (amino acid 111) may represent a

common polymorphic site in the human kallikrein gene [40].

Construction of expression plasmid pKA21

Initial experiments to express mature kallikrein in a cytoplasmic bound form revealed that the expression product was associated with the membrane fraction of *E. coli*. Hydrophobicity-plot analysis showed that kallikrein contains two hydrophobic domains which could be responsible for the finding that cytoplasmically expressed kallikrein was only found in the membrane fraction. Therefore we decided to construct an expression plasmid in which the nucleotide sequence of mature kallikrein starting with +1 isoleucine is fused in frame to the signal peptide of alkaline phosphatase. This signal peptide should direct the gene product into the periplasmic space of *E. coli*. In the construction the complete prepro sequence was deleted because additional N-terminal amino acids of the kallikrein pro sequence could result in a proteolytically inactive protein. As expression vector we chose pSP6 [34], a derivative of the expression vector pKK223-3 [41] which carries the *tac* promoter and the Shine-Dalgarno sequence upstream of a multipurpose cloning site and, in addition, a ribosomal RNA transcription terminator downstream of this site. This vector has successfully been used to express human proteinase inhibitors [42]. The construction of the final plasmid pKA21 for expression and secretion of human salivary-gland kallikrein is detailed in Fig. 3. The first stage was to delete the poly(A) and poly(C) sequence present in the 3'-untranslated part of the cDNA. This was achieved by isolating the 940 bp *Pst*I fragment and by subsequently generating a cDNA fragment lacking the codons for the ten C-terminal amino acids by partial digestion with *Sau*3A. This fragment was subcloned together with a synthetic DNA fragment (AN1, AN2, Fig. 3) in *Pst*I/*Hind*III-hydrolysed pUC18 to give the recombinant plasmid pKA18. In this construction the synthetic DNA fragment completes the previously deleted ten C-terminal amino acids, including two translational stop codons. In order to delete the 5'-untranslated region and the prepro-sequence in the kallikrein cDNA plasmid, pKA18 was hydrolysed with *Hind*III/*Rsa*I and ligated together with a second synthetic DNA fragment (AN5, AN6; Fig. 3) in the expression vector pKK223-3, which was hydrolysed with *Eco*RI and *Hind*III. The resulting expression plasmid pKA21 now contains the coding cDNA sequence of the mature salivary-gland kallikrein fused in frame to the signal peptide sequence of alkaline phosphatase under the control of the *tac* promoter.

Fig. 3. Construction of the secretion plasmid pKA21

Black arrow, *tac* promoter (P_{tac}); hatched part, cDNA encoding kallikrein; cross-hatched part, oligonucleotides AN₁ (5'-GATCGAGGACACCATAGCGGAGAACTCCTAATGATATCA3') and AN₂ (5'-AGCTTGATATCATTAGGAGTTCCTCGCTATGGTGTCTCTC3') for reconstitution of the C-terminus; dotted part, oligonucleotides AN₅ (5'-AATTGTGAAACAAAGCACTATTGCACTGGCACTCTTACCGTTACTGTTTACCCCTGTGACAAAAGCAATCGTTGGTG-GCTGGGAATGCCTTGTCCTACTCTCAGCCGTGGCAGGCTGCTCTGT3') and AN₆ (5'-ACAGAGCAGCTCGCCACG-GCTAAGAGTGGACAAGGCATTCCCAGCCACCAACGATTGCTTTTGTACAGGGGTAAACAGTAACGGTAAG-AGTGCCAGTG 3') coding for the alkaline phosphatase leader peptide and the amino acids +1 to +18 of kallikrein given in Fig. 2. The open box indicates transcription terminator (*rrnBT₁T₂*). E, H, R, S and P, restriction enzymes *Eco*RI, *Hind*III, *Rsa*I, *Sau*3A and *Pst*I.

Fig. 4. Synthesis of human kallikrein in *E. coli* BMH 71-18

A Western-blot analysis is shown. Periplasmic proteins from *E. coli* corresponding to 2 A_{578} units harbouring either plasmid pKA21 or pSP6 [34] were subjected to SDS/12.5%-PAGE and transferred to nitrocellulose. Incubation with anti-kallikrein antiserum was done as described in the Materials and methods section. (a) Periplasmic proteins of *E. coli* BMH 71-18/pSP6; (b) periplasmic proteins of *E. coli* BMH 71-18/pKA21. Numbers on the top indicate different times of sample collection; 0, before induction; lanes 1–5, 0.5, 1, 2, 3 and 16 h after induction with IPTG; lane R, 400 ng of human urinary kallikrein; lane S, molecular-mass (—) markers [ovalbumin (45 kDa), α -chymotrypsinogen (24 kDa) and β -lactoglobulin (18 kDa)].

Expression and characterization of recombinant kallikrein

To verify kallikrein expression, two highly sensitive methods were applied. Western-blot analysis with antibodies directed against human urinary kallikrein was performed, and in a second approach specific proteolysis of the substrate acetylphenylalanylalanyl ethyl ester was monitored as described by Fiedler *et al.* [37]. By using specific IgGs, expression of kallikrein could be detected 30 min after induction. The rate of production was constant over a period of 8 h, with a subsequent decline 12 h after induction (Fig. 4). The amount of the recombinant kallikrein expressed 3 h after induction was estimated to be in the range of 200–500 $\mu\text{g/l}$ of culture or 0.7–1.5 $\mu\text{g}/A_{578}$ unit. This estimate of kallikrein production was based on Western blots obtained with urinary kallikrein as a reference. Interestingly, bacteria transformed by pKA21 showed reduced growth 8 h after induction, suggesting that the expression of kallikrein interferes with essential biological processes in *E. coli*. This growth inhibition may also explain the decline of expressed kallikrein 12 h after induction. The difference in molecular mass between recombinant kallikrein and urinary kallikrein seen after SDS/PAGE is due to the glycosylation of urinary kallikrein in contrast with the unglycosylated form of the *E. coli* expression product.

We also measured the kallikrein activity by using a coupled two-enzyme assay. By using different amounts of the osmotic-shock fluids of induced *E. coli* BMH 71-18/pKA21, significant increase in proteolytic activity was measured against induced *E. coli* BMH 71-18/pSP6 as a control (Fig. 5). The amount of proteolytically active kallikrein produced is at a level of about 3 munits/ A_{578} cells or 3 ng/ A_{578} cells. This value is calculated on the basis of monitoring the activity of isolated urinary kallikrein under identical conditions. A comparison of these amounts of proteolytically active recombinant kallikrein with those seen in Western-blot analysis shows that only about 1% of the expressed kallikrein is proteolytically active. This observation could be ex-

Fig. 5. Proteolytic activity of recombinant human kallikrein

At 2 h after induction, different amounts of the periplasmic fraction of *E. coli* BMH 71-18 harbouring the recombinant plasmid pKA21 (□) or pSP6 (●) were tested. Proteolytic activity was measured as described by Fiedler *et al.* [37].

plained by the fact that kallikrein contains ten cysteine residues which are all involved in the formation of disulphide bonds and of the correct three-dimensional structure to expose the active site. Incorrect formation of the disulphide bonds would probably lead to proteolytically inactive protein. Although we have used an expression system that directs its product into the periplasmic space, it does not ensure the formation of the correct disulphide bonds. On the other hand, the expression product detected by specific anti-kallikrein IgGs in Western-blot analysis may consist of a heterogeneous population of differently processed forms of kallikrein which are not separated by SDS/PAGE (Fig. 4). It can be assumed that kallikrein with an incorrectly processed N-terminus is not proteolytically active.

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REFERENCES

- Müller-Esterl, W., Iwanaga, S. & Nakanishi, S. (1986) *Trends Biochem. Sci.* **11**, 336–339
- Fiedler, F. (1979) *Handb. Exp. Pharmacol.* vol. **25** (Suppl.), 103–161
- Regoli, D. & Barabe, J. (1980) *Pharmacol. Rev.* **32**, 1–46
- Wicklmayer, M., Dietze, G., Mayer, L., Böttger, T. & Grunst, J. (1979) *FEBS Lett.* **98**, 61–65
- Schill, W. B., Preissler, G., Dittmann, B. & Müller-Esterl, W. (1979) *Adv. Exp. Med. Biol.* **120**, 305–310
- Fiedler, F., Ehret, W., Godec, G., Hirschauer, C., Kutzbach, C., Schmidt-Kastner, G. & Tscheche, H. (1977) in *Kininogenases 4* (Haberland, G. L., Rohen, J. W. & Suzuki, T., eds.), pp. 7–14, Schattauer, New York, Stuttgart
- Ekfors, T. O. & Hopsu-Havu, V. K. (1971) *Histochem. J.* **3**, 415–420
- Chao, J., Chao, L. & Margolius, H. S. (1984) *Biochim. Biophys. Acta* **801**, 244–249
- Chao, J., Woodley, C. M., Chao, L. & Margolius, H. S. (1983) *J. Biol. Chem.* **258**, 15173–15178
- Nolly, H., Scili, A. G., Scili, G. & Carretero, O. A. (1985) *Circ. Res.* **56**, 816–821
- MacDonald, R. J., Margolius, H. D. S. & Erdős, E. G. (1988) *Biochem. J.* **253**, 313–321
- Fukushima, D., Kitamura, N. & Nakanishi, S. (1985) *Biochemistry* **24**, 8037–8043
- Lottspeich, F., Geiger, R., Hemshen, A. & Kutzbach, C. (1979) *Hoppe-Seyler's Z. Physiol. Chem.* **262**, 1947–1950
- Berger, E. A. & Shooter, E. M. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3647–3651
- Frey, P., Forand, R., Maciag, T. & Shooter, E. M. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 6290–6298
- Evans, B. A., Drinkwater, C. C. & Richards, R. J. (1987) *J. Biol. Chem.* **262**, 8027–8034
- Gerald, W. L., Chao, J. & Chao, L. (1986) *Biochim. Biophys. Acta* **866**, 1–14
- Howles, P. N., Dickinson, D. P., DiCaprio, L. L., Woodworth-Gutai, M. & Gross, K. W. (1984) *Nucleic Acids Res.* **12**, 2791–2805
- Baker, A. R. & Shine, J. (1985) *DNA* **4**, 445–450
- Schedlich, L. J., Bennetts, B. H. & Morris, B. J. (1987) *DNA* **6**, 429–437
- Watt, K. W. K., Lee, P.-J., M'Timkulu, T., Chan, W.-P. & Loo, R. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3166–3170
- Lundwall, A. & Lilja, H. (1987) *FEBS Lett.* **214**, 317–322
- Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299
- Gubler, U. & Hoffman, B. J. (1983) *Gene* **25**, 263–269
- Hanahan, D. & Meselson, M. (1980) *Gene* **10**, 63–67
- Thayer, R. E. (1979) *Anal. Biochem.* **98**, 60–63
- Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: a Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
- Wallace, R. B., Johnson, M. J., Hirose, T., Miyatze, T., Kawashima, E. H. & Itakura, K. (1981) *Nucleic Acids Res.* **9**, 879–894
- Denhardt, D. T. (1966) *Biochem. Biophys. Res. Commun.* **23**, 641–646
- Hardies, S. C., Patient, R. K., Klein, R. D., Ho, F., Reznikoff, W. S. & Wells, R. D. (1979) *J. Biol. Chem.* **254**, 5527–5534
- Korneluk, R. G., Quan, F. & Gravel, R. A. (1985) *Gene* **40**, 317–323
- Maxam, A. & Gilbert, G. (1980) *Methods Enzymol.* **65**, 499–580
- Evans, B. A., Xiao Yun, Z., Close, J. A., Tregear, G. W., Kitamura, N., Nakanishi, S., Callen, D. F., Baker, E., Hyland, V. J., Sutherland, G. R. & Richards, R. I. (1988) *Biochemistry* **27**, 3124–3129
- Dodt, J., Schmitz, T., Schäfer, T. & Bergmann, C. (1986) *FEBS Lett.* **202**, 373–377
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Fiedler, F., Geiger, R., Hirschauer, C. & Leysath, G. (1978) *Hoppe-Seyler's Z. Physiol. Chem.* **259**, 1667–1673
- Proudfoot, N. J. & Brownlee, G. G. (1976) *Nature (London)* **263**, 211–214
- Proudfoot, N. J. (1984) *Nature (London)* **307**, 412–413
- Maniatis, T., Fritsch, E. F., Lauer, J. & Lawn, R. M. (1980) *Annu. Rev. Genet.* **14**, 145–178
- DeBoer, H. A., Comstock, L. J. & Vasser, M. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 21–25
- Strauss, M., Bartsch, F. O., Stollwert, J., Trstenjak, M., Böhning, A., Gassen, H. G., Machleidt, W. & Turk, V. (1988) *Biol. Chem. Hoppe-Seyler* **369**, 209–218

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