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The isolation and partial characterization of a new restriction endonuclease from Providencia stuartii.

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ABSTRACT

We describe the isolation of a new class 2 restriction endonuclease from Providencia stuartii 164. Using the procedure of osmotic shock treatment, we have partially purified this enzyme (Pst 1) and have begun preliminary work to characterize its specificity and requirements. Pst 1 requires Mg^{++} as the only cofactor and produces more than 18 cleavages in wild type lambda. We have determined the location of 7 of these cleavages by the use of deletion and insertion mutants of lambda.

INTRODUCTION

Since their discovery, restriction endonucleases have become invaluable tools in research; their use in the analyses of DNAs from both procaryotes and eucaryotes has been well documented. They have been found useful in such diversified problems as the elucidation of rDNA structure in Xenopus Laevis[1] and structural comparisons between bacterial plasmids in E. coli[2]. More recently, they have been used to clone procaryotic and eucaryotic genes by transformation[3,4].

This paper describes the partial purification of a restriction endonuclease from a clinical isolate, Providencia stuartii 164 utilizing the procedure of osmotic shocking first described by Neu and Heppel[5]. The osmotic shocking procedure releases only those proteins that are located in the periplasmic space and does not release internal proteins[6]. Pst 1 was not found in other strains of Providencia stuartii that we have examined which suggests that it is a plasmid coded enzyme.

Pst 1 was conveniently prepared by osmotic shock treatment of Tris-EDTA treated cells, followed by chromatography on DEAE cellulose and phosphocellulose. The enzyme produces more than 18 cleavages in wild type lambda DNA, and we have determined the location of 7 of these.

MATERIALS AND METHODS

Chemicals. DEAE cellulose (medium grade) was obtained from Sigma Chemical Company, and phosphocellulose P-11 was obtained from Whatman. Glycerol was

obtained from Fisher Scientific (It is important not to use glycerol obtained from metal containers), agarose from Miles Chemical, and bromphenol blue and ethidium bromide from Allied Chemical. Acrylamide was obtained from Eastman Kodak.

Strains. The strains of bacteria and bacteriophages employed in this study are listed in Table 1. Phage DNA was purified by phenol extraction.

Table 1

Strains	Source (reference)
Bacteria:	
E. coli RY 13	Weisblum, B. (7)
Providencia stuartii 164	Uwaydah, M. (8)
phages:	
lambda plac 5	Reznikoff, W. (9)
lambda b189	Parkinson, S. (10)
lambda b515	Parkinson, S. (10)
lambda b511	Parkinson, S. (10)
lambda b519	Parkinson, S. (10)
lambda b1319	Henderson, D. (11)
lambda KH100 nin 5	Blattner, F. (12)
lambda KH70	Blattner, F. (12)
lambda imm 434	Blattner, F. (12)

Pst 1 Purification. Cells were grown in 10 l of a medium containing 10 g bacto-tryptone (Difco), 5 g yeast extract (Difco) and 10 g NaCl per l to an OD_{550} of 1.1 at 37C, and harvested by centrifugation at 16,000 X G for 10 min. Cells were washed twice with 175 ml of a buffer containing 0.01 M Tris-HCl pH 7.8, 0.03 M NaCl, resuspended in 175 ml of a buffer containing 0.033 M Tris-HCl pH 7.8, 0.003 M EDTA, and 20% sucrose and stirred vigorously at room temperature with a magnetic stirring bar for 20 min. After centrifugation at 16,000 X G for 10 min, the supernatant was discarded and excess sucrose was removed from the tube and pellet with a cotton swab. The pellet was then resuspended in 100 ml ice cold 5 X 10^{-4} M $MgCl_2$, stirred vigorously (magnetic stirring bar) at 4C for 10 min and centrifuged at 16,000 X G for 10 min. The supernatant, designated the osmotic shockate, was adjusted to 0.01 M $NaPO_4$ pH 7.0, 0.001 M EDTA, 0.001 M β-mercaptoethanol and 0.15% Triton X-100 (Buffer A) by addition of concentrated stock solutions and applied to a DEAE column (1.5 x 20 cm). The enzyme was eluted with a 500 ml gradient from 0 - 0.5 M NaCl in Buffer A.

Fractions were assayed for endonuclease activity by electrophoresis after digestion of lambda DNA. The active fractions (eluting at 0.14 - 0.28 M NaCl) were pooled (100 ml) and diluted fivefold with Buffer A. The diluted enzyme was applied to a phosphocellulose column (1.5 x 20 cm) that had previously been equilibrated with Buffer A and 0.05

M NaCl. The enzyme was eluted with a 500 ml gradient from 0.05 - 0.6 M NaCl. Fractions containing endonuclease activity (eluting at 0.34 - 0.41 M NaCl) were pooled (45 ml) and dialyzed overnight against Buffer A plus 50% glycerol (v/v); this treatment resulted in a threefold concentration. The enzyme was stored at -20C in small portions.

Small Scale Osmotic Shockates. Rapid analysis of endonuclease activity of 100 ml cultures was carried out as follows: 100 ml cultures were grown at 37C to an OD_{550} of 1.1. Cells were harvested and osmotically shocked as was described in Pst 1 purification except that 3 ml of cold 5×10^{-4} M $MgCl_2$ was added in the last step. Osmotic shockates containing endonuclease activity are stable for months at -20C.

EcoRI Purification. EcoRI was purified as previously described.[7]

Digestion Procedure 1. To 0.1 - 0.2 micrograms of DNA was added 5 microliters of 10X salts (0.9 M Tris-HCl pH 7.4, 0.1 M $MgSO_4$), 5 microliters of endonuclease solution, and water to 50 microliters. The mixture was incubated at 30C for 20 min and the reaction stopped by the addition of 25 microliters of a solution containing 0.05 M EDTA, 0.01% bromphenol blue, and 30% sucrose.

Electrophoresis Procedure 1 was used for the assay of restriction endonuclease activity in chramotographic fractions. 8 mm x 15 cm tubes were used for electrophoresis; they have a small constriction at the bottom to prevent the gels from slipping out. The tubes were filled with 3 ml of 1% agarose in electrophoresis buffer (Tris 0.04 M, sodium acetate 0.2 M, EDTA 0.01 M (adjusted to pH 7.0 with glacial acetic acid) and 0.5 micrograms/ml EtBr). The incubation mixture (75 microliters) was applied to the top of the gel and electrophoresed at 3 mA per tube until the band of bromphenol blue reached the bottom of the gel tube (usually 3-1/2 hr). The gels were then removed from the tubes, and the DNA fragments detected as fluorescent bands on exposure to UV light.

Digestion Procedure 2. This procedure is identical to digestion procedure 1 except that in this case 1.0 - 3.0 micrograms of DNA were digested with 20 microliters of purified endonuclease. This procedure was used with the purified enzyme to obtain DNA fragments in order to ascertain sites of cleavage in phage lambda.

Electrophoresis Procedure 2. This procedure was similar to that described for electrophoresis in 1% agarose in tubes[13], modified for use with slab gels[14]. In this procedure, however, only 15 microliters of final enzyme mixture (corresponding to 0.5 - 1.0 micrograms of DNA) was applied to the gel.

Polyacrylamide Gels. Polyacrylamide gels were prepared and used as previously described[15].

Preparation of Cell Extracts by Sonication. Cells were exposed to two 30 sec bursts at 75 watts in a small chilled glass tube with a Sonifier Cell Disruptor. The unbroken cells, debris, and ribosomes were removed by centrifugation at 100,000 X G for 90 min. The supernatant is referred to as the sonicate.

RESULTS

Osmotic Shocking as a Means of Screening Gram Negative Bacteria for the Presence of Restriction Endonucleases. We have found osmotic shocking on a small scale to be a useful way of screening bacteria for the presence of restriction endonucleases. Using this technique we have isolated three previously unreported restriction endonucleases in clinical isolates of Providencia stuartii, Klebsiella pneumoniae, and Moraxella glueidi. This paper describes the work carried out with the restriction endonuclease Pst 1 isolated from Providencia stuartii 164.

To determine whether osmotic shocking is an effective method of releasing all of the restriction endonuclease component of the cell, we prepared osmotic shockates of Providencia stuartii 164 and Eschericia coli RY 13, and in addition, sonicated the pellets remaining after the 5×10^{-4} M $MgCl_2$ step to release enzymes not released by osmotic shocking. By digestion and electrophoresis, we compared the restriction activity of the crude osmotic shockates to that remaining in the cells after the osmotic shocking step, released by sonic disruption. These results are seen in Figure 1. The osmotic shockates clearly contain discernible restriction endonuclease activity but the total restriction endonuclease complement of both Providencia stuartii 164 and Eschericia coli RY 13 is not released by the osmotic shock procedure since considerable restriction activity is released from the cells by sonication after osmotic shock treatment. In spite of this incomplete release the technique of osmotic shock treatment of cells provides a convenient small scale isolation and partial purification procedure for restriction endonucleases. The enzymes are not pure and contaminating nucleases are present in preparations from certain strains, these are detected after incubations of long duration (>1 hr) when bands lose their sharpness in gel electrophoresis. $EcoR_I$ preparations from E. coli RY 13 are relatively free of nuclease contamination since this strain lacks endonuclease I, also released by osmotic shocking.

Pst 1 Purification. The new restriction enzyme Pst 1 was purified as described in Materials and Methods. Digestion patterns obtained with the

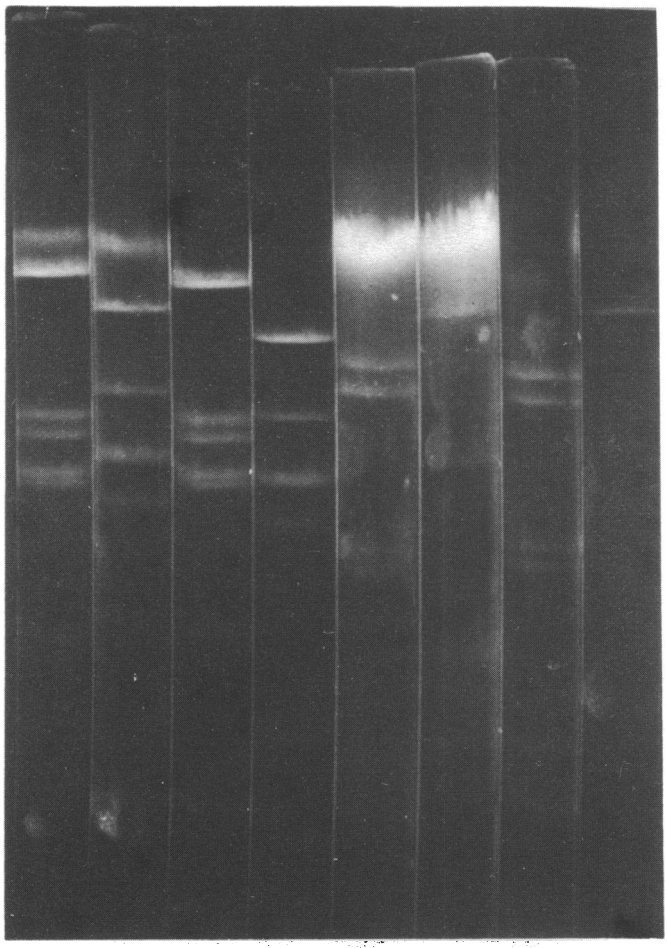

Fig. 1. (From left to right)
1. lambda plac 5 after digestion with Providencia stuartii 164 osmotic shockate.
2. phi80 plac after digestion with Providencia stuartii 164 osmotic shockate.
3. lambda plac 5 after digestion with Providencia stuartii 164 sonicate from osmotically shocked cells.
4. phi80 plac after digestion with Providencia stuartii 164 sonicate from osmotically shocked cells.
5. lambda plac 5 after digestion with E. coli RY 13 osmotic shockate.
6. phi80 plac after digestion with E. coli RY 13 osmotic shockate.
7. lambda plac 5 after digestion with E. coli RY 13 sonicate from osmotically shocked cells.
8. phi80 plac after digestion with E. coli RY 13 sonicate from osmotically shocked cells.
Enzyme preparation and digestion are as described in Materials and Methods.

crude osmotic shockate and with the purified enzyme preparation are shown in Figure 2. For comparison the analogous EcoRI digestion patterns are shown. Clearly the major if not the only endonuclease activity in the osmotic shockate of Providencia stuartii 164 has been purified.

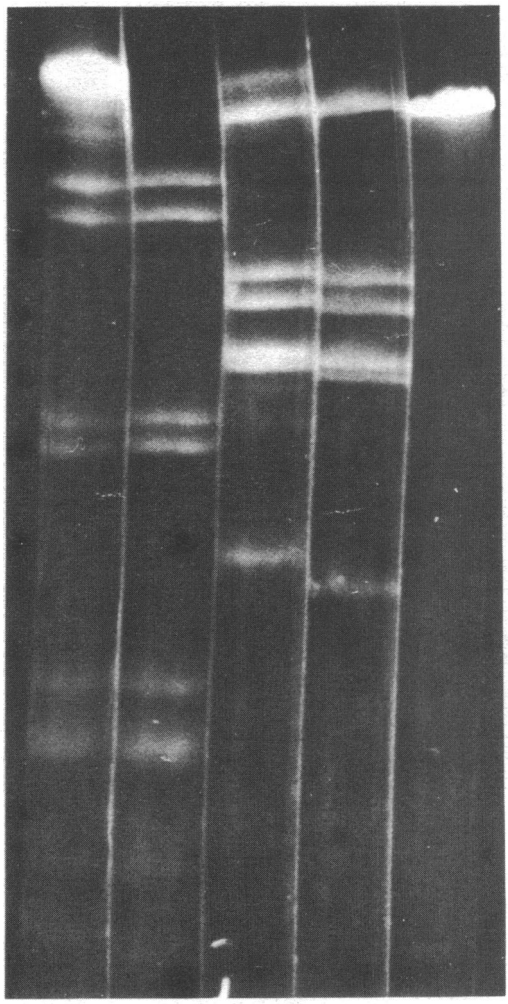

Fig. 2. (From left to right)
1. lambda plac 5 digested with a crude osmotic shockate of Providencia stuartii 164.
2. lambda plac 5 digested with the purified Pst 1 enconuclease.
3. lambda plac 5 digested with a crude osmotic shockate of E. coli RY 13.
4. lambda plac 5 digested with purified EcoRI.
5. undigested lambda plac 5.
Enzyme preparation and digestion methods are as described in Materials and Methods.

The purification of the endonuclease was monitored by polyacrylamide gel electrophoresis. An osmotic shockate preparation of Providencia stuartii 164 endonuclease contained more than 30 protein bands as assayed by gel electrophoresis, whereas the partially purified enzyme preparation contained only 5 protein bands. A similar preparation of EcoRI endonuclease from E. coli RY 13 showed essentially analogous results.

Properties of Pst 1. The enzyme appears to cleave DNA efficiently at very low salt concentrations. As the NaCl concentration was increased, the ability to digest DNA decreased and above 0.3 M NaCl there was no detectable digestion of DNA.

In the absence of Mg^{++} Pst 1 was unable to cleave DNA; this appears to be the only required co-factor. Endonuclease Pst 1 was unstable to heat, whereas EcoRI was stable for 2 min at 50C; Pst 1 showed almost complete loss of activity after heating for 2 min at 37C. Since the enzymes is not pure we cannot say if this is an intrinsic property of the enzyme or is due to a contaminating protease however all digestions with endonuclease Pst 1 when carried out at 30C gave reproducible results.

The Pst 1 activity present in osmotic shockates of Providencia stuartii 164 was stable at -20C for at least six months. This was not the case with the more purified enzyme which rapidly loses endonuclease activity over a period of several weeks at -20C.

Sites of Cleavage in Phage Lambda. Figure 3 shows a map of lambda wild type and the deletion and insertion mutants of lambda that were used to examine sites of cleavage in lambda by endonuclease Pst 1. The location and size of each deletion or insertion is known from analysis of heteroduplexes in the electron microscope.

Each of the DNA preparations was digested with endonuclease Pst 1 and the reaction mixtures were electrophoresed on agarose slab gels in the presence of molecular weight standards. The size of each fragment was determined by reference to a calibration curve, and changes in the banding patterns of each of the mutants relative to lambda wild type were correlated with the known structure of the mutants.

Endonuclease Pst 1 produces more than 18 fragments in lambda wild type, some of which are too small for proper resolution in 1% agarose. The pattern of digestion for each of the lambda mutants is illustrated schematically in Figure 4. (However, we have not included fragments less than 4% of the length of lambda due to their poor resolution in our system).

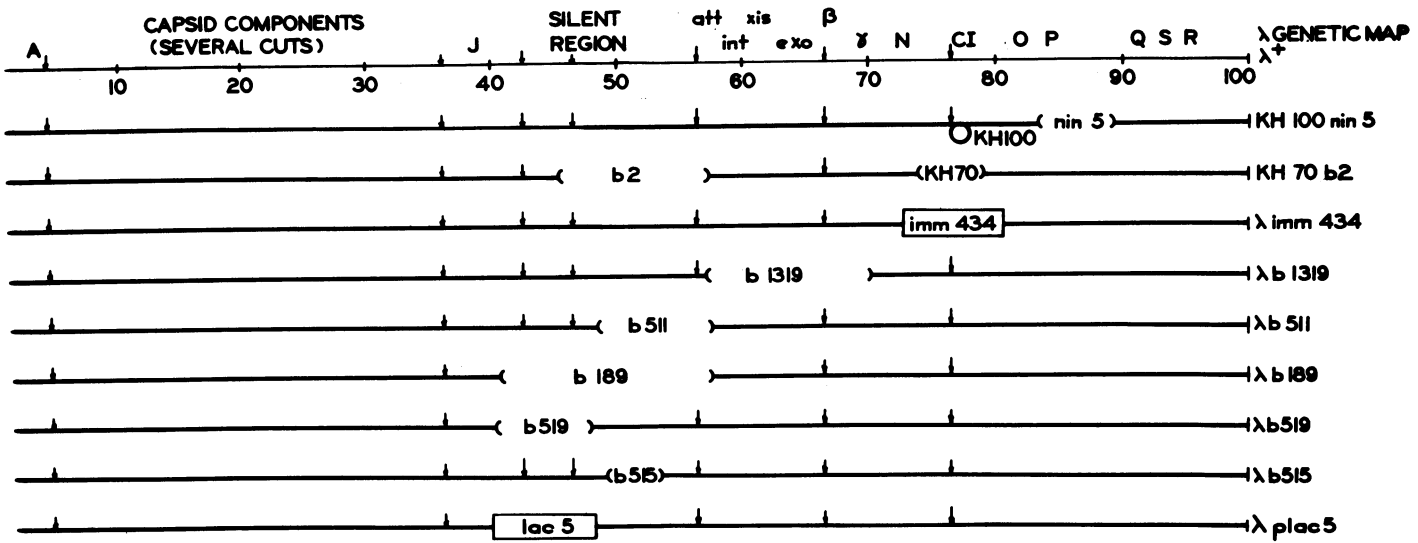

Fig. 3. Map of lambda wild type DNA and the deletion and insertion mutants used to position 7 sites of cleavage in lambda wild type. Arrows indicate sites of cleavage by Pst 1.

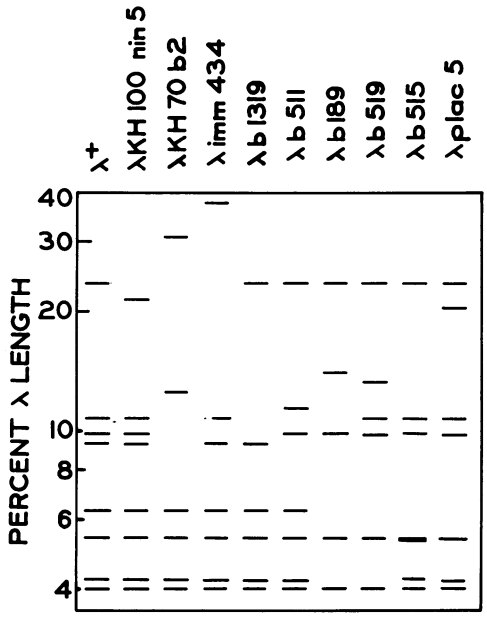

Fig. 4. Schematic representation of digestion patterns produced by Pst 1 endonuclease. Fragments with a molecular weight less than 4% of the length of lambda are not shown.

There is little difficulty in assigning the positions of the eight largest fragments on the lambda map.

The largest fragment in the digest is at the right hand end, and the cleavage producing it is within the immunity regions, since KH 70 creates a large fusion fragment. The fragment at the left hand end measures 4.2% of the length of lambda DNA and was characterized by the fact that when the digestion mixture was heated to 55C and then quickly chilled a new band corresponding to 4.2% of the length of lambda appears. In the absence of heating this band is barely visible. We therefore assume that the fragment contains the left hand cohesive end of lambda DNA.

Five more large fragments are located in the right two-thirds of the map in the order shown in Figure 3. A 5% fragment produced by the Pst 1 endonuclease was found when each of the mutants was digested and must therefore lie in the region between 4.2% and 36% on the lambda map. The smaller fragments pose difficulty of assignment, particularly in the region between 4.2% and 36% on the lambda map since we had no deletion mutants available to define this region.

DISCUSSION

We have shown that osmotic shocking is a useful way to screen gram-negative bacteria for the presence of restriction endonucleases. By this method we have detected a previously unreported restriction endonuclease in a clinical isolate of Providencia stuartii. Subsequent studies have demonstrated new restriction endonucleases in clinical isolates of Klebsiella pneumoniae, and Moraxella glueidi[16]. Enzyme purification is greatly simplified when starting from an osmotic shockate since such preparations contain only 30-100 proteins[6]. However, one drawback in using osmotic shockates is that they do not appear to release all of the restriction endonuclease activity of E. coli RY 13 and Providencia stuartii 164. The use of sonication in screening for new restriction endonucleases is complicated because extracts prepared by sonication often contain non-specific nucleases which can "mask" the presence of a restriction endonuclease.

Instability has been the major problem with endonuclease Pst 1; the enzyme is less effective at 37C than at 30C. A crude osmotic shockate of Providencia stuartii 164 gives clearly discernible digestion of lambda wild type DNA even after storage for six months at -20C. However, more highly purified preparations of enzyme usually lose activity within 3 to 4 weeks, despite efforts to stabilize the enzyme.

Nevertheless, we have been able to locate the position of seven of the cleavages produced by Pst 1 on the lambda map; lambda DNA is cleaved more then 18 times by Pst 1, leaving at least 11 other points of cleavage to be characterized. We have not yet determined if Pst 1 cleavages produce "sticky-end" fragments nor do we know the sequence of DNA recognized by the Pst 1 endonuclease.

The positions of cleavage in SV40 and in polyoma produced by Pst 1 are known[17]; SV40 is cleaved twice at 0.04 and 0.275 on the map. Polyoma is cleaved 5 times at 80 (in Hpall-4), 51 (Hpall-1), 32.5 (Hpall-1), 16 (Hpall-2), and 14.5 (Hpall-2).

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REFERENCES

1. Wellauer, P., Reeder, R., Carroll, D., Brown, D., Deutch, A., Higashinakagawa, T. (1974) Proc. Nat. Acad. Sci. USA 71, 2823
2. Thompson, R., Hughes, S., Broda, P. (1974) Molec. and Gen. Genetics 133, 141
3. Cohen, S., Chang, A., Boyer, H., Helling, R. (1973) Proc. Nat. Acad. Sci. USA 70, 3240
4. Morrow, J., Cohen, S., Chang, A., Boyer, H., Goodman, H., Helling, R. (1974) Proc. Nat. Acad. Sci. USA 71 1743
5. Neu, H. and Heppel, L. (1965) J. Biol. Chem. 240, 3685
6. Heppel, L. A. (1971) "The concept of periplasmic enzymes", from Structure and Function of Biological Membranes edited by L. C. Rothfield, Academic Press
7. Weisblum, B. and Tanaka, T. (1975) J. Bact. 121, 354
8. Chevereau, M., Daniels, P., Davies, J., LeGoffic, F. (1974) Biochemistry 13, 598
9. Ippen, K., Shapiro, J., Beckwith, J. (1971) J. Bact. 108, 5
10. Parkinson, J., Huskey, R. (1971) J. Mol. Biol. 56, 369, 385
11. Henderson, D. and Weil, J. (1975) Genetics 79, 143
12. Blattner, F., Fiant, M., Hass, H., Twose, P., Sybalski, W. (1974) Virology 62, 458
13. Helling, R., Goodman, H., Boyer, H. (1974) J. Virol. 14, 1235
14. Shinnick, T., Lund, E., Blattner, F., Smithies, O. (1974) Nucleic Acids Research (in press)
15. Hedrick, J., Smith, A. (1968) Archives Biochem. and Biophys. 126, 155
16. Smith, D., unpublished results
17. Crawford, L. V., Robbins, A. K. (submitted for publication)