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A Systematic DNA Sequencing Strategy

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A systematic DNA sequencing strategy is presented. Instead of the sequencing of randomly selected DNA fragments (the "shotgun" method), the nucleotide order is progressively determined along a DNA chain using the dideoxynucleotide termination sequencing system and the single-stranded bacteriophage vector M13 derivatives. The length of DNA along which the sequence data can be progressively read appears to be limited only by the insertion capacity of the vector. As an example of this strategy a recombinant replicative form with a 2327 nucleotide long *Hin*dIII fragment from the restriction enzyme digest of bacteriophage λ DNA was prepared. The replicative form of the recombinant was partially digested with DNAase I in the presence of Mn^{2+}. As the replication origin of the phage vector was located near one end of the inserted DNA and the priming site of the vector at the other, the breaks outside the inserted DNA either destroyed the phage or removed the priming site. With the use of a unique restriction site close to the priming site, the breaks within the inserted DNA gave rise to a recombinant mixture with the inserted DNA fixed at one end and sequentially shortened at the other. Using the ddT reaction screening procedure, 11 recombinants were identified in which the inserted DNA varied in length by about 200 nucleotides. Sequencing these recombinants by the dideoxynucleotide sequencing system covered the whole 2327 nucleotides of the *Hin*dIII DNA fragment. The average number of nucleotides read from a gel was 210, which fell into the most readable region of a sequencing gel: the overlapping regions between two gels were of 33 to 48 nucleotides.

1. Introduction

DNA sequencing has become one of the most important tools for the analysis of genes. Two fast and reliable DNA sequencing systems (Maxam & Gilbert, 1977; Sanger *et al.*, 1977) are available that allow the sequence determination of a stretch of DNA up to about 400 nucleotides (Sanger & Coulson, 1978). Longer sequences have to be determined in short stretches, which are then joined together on the basis of their overlaps.

A random DNA sequencing strategy (the "shotgun" method) has been developed recently using the dideoxynucleotide chain termination sequencing system and single-stranded bacteriophage vector M13 derivatives (Gronenborn & Messing,

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1978; Heidecker *et al.*, 1980; Sanger *et al.*, 1980; Anderson, 1981; Messing *et al.*, 1981). A similar approach has been used by the Maxam–Gilbert method (Rüther *et al.*, 1981). Combined with computer programs (Gingeras *et al.*, 1979; Staden, 1979,1980), the shotgun strategy allows the rapid accumulation of sequence data. However, as this is a random system, towards the end of an investigation new data will accumulate more slowly and will be accompanied by an increasing amount of repetitive data.

A systematic DNA sequencing strategy is presented whereby it is possible to determine the nucleotide order progressively along a DNA chain.

2. Principle of the Strategy

The principle of the strategy is summarized in Figure 1. The replicative form of an M13 vector derivative was prepared with a unique restriction enzyme recognition site (A) close to the priming site of the vector, and another unique restriction site (B) as an insertion site within the β-galactosidase gene. The DNA to be sequenced was inserted in the vector at B using the corresponding restriction enzyme. The recombinant DNA thus prepared was partially digested with DNAase I in the presence of Mn^{2+}. The breaks within the inserted DNA gave rise to a mixture of linear double-stranded recombinants with varying lengths of inserted DNA adhering to both ends. Treatment of the mixture with a corresponding restriction endonuclease removed the DNA pieces adjoining the priming site. Circularization of these linear recombinants with T4 DNA ligase produced a clone mixture with insert DNA fixed at one end and sequentially shortened at the other close to the priming site. As clones with longer inserts diffuse more slowly, plaques of various size representing different lengths of insert DNA were formed on the agarose plate. The plaques of varying sizes were then picked and single-stranded phage DNA was prepared. Using a slight modification of the ddT reaction screening procedure (Sanger *et al.*, 1980), clones in which the inserted DNA varied in length by about 200 base-pairs were identified; these clones covered the whole stretch of the insert DNA and were sequenced by the dideoxynucleotide termination method (Sanger *et al.*, 1977).

During the digestion with DNAase I (stage (d), Fig. 1) breaks will occur randomly around the recombinant, but the sections ultimately sequenced will be those that are derived from breaks within the insert. Since the origin of replication of the vector is close to the left-hand end (Fig. 1) of the insert (Schaller *et al.*, 1978), breaks on this side will lead to molecules without an origin (stage (e), Fig. 1) that do not give viable phage, and breaks on the other side of the insert will give phage that contains no priming site (Duckworth *et al.*, 1981) and therefore cannot be sequenced. A few clones were obtained that were smaller than the original vector: these "nonsense" clones were probably caused by DNAase breaks on the vector near the ends of the insert.

The following section describes the application of the method to a 2327-nucleotide *Hin*dIII fragment from bacteriophage λ (fragment 1) that is located in the b region between positions 25,174 and 27,501 (F. Sanger, A. R. Coulson, G. F. Hong, D. Hill & G. B. Petersen, unpublished data).

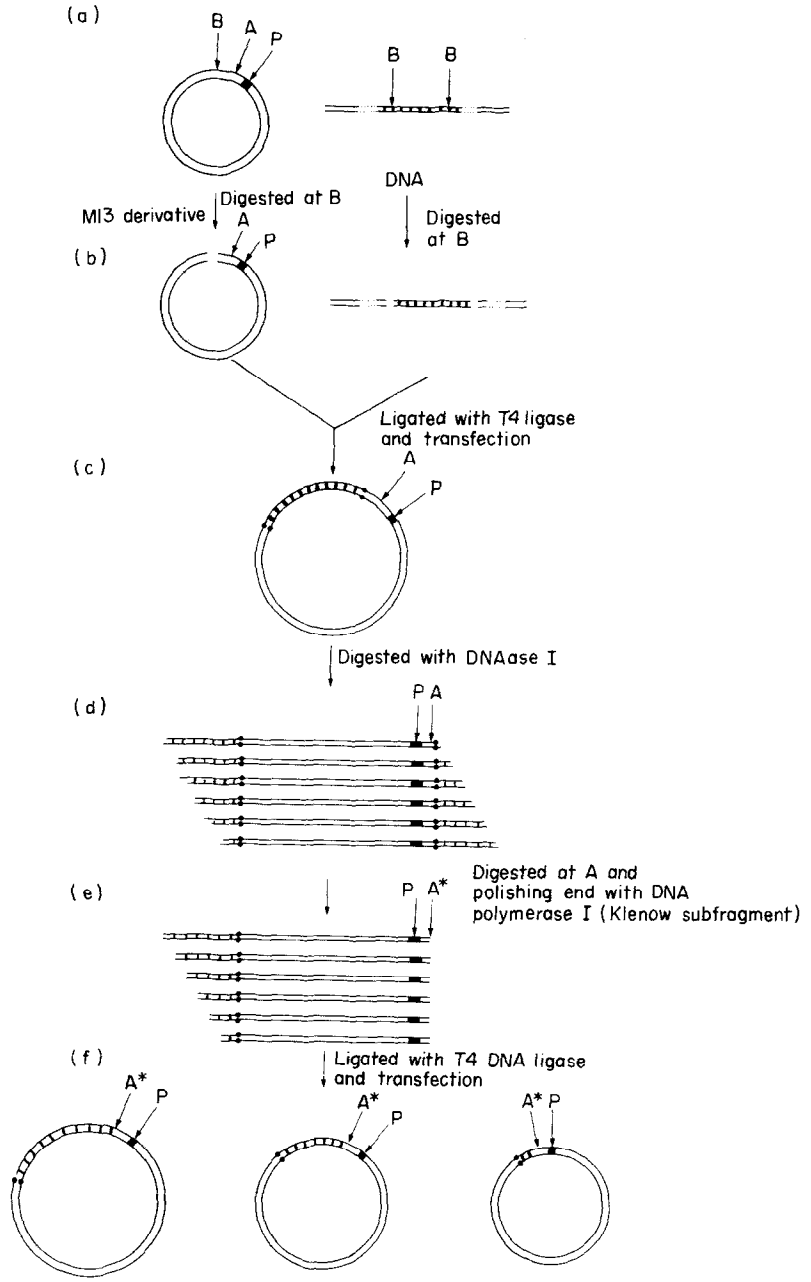

FIG. 1. Diagram illustrating the systematic strategy for DNA sequencing. A, a unique restriction endonuclease recognition site, giving a flush end or recessed sticky end after digestion. B, any unique DNA restriction site for the insertion of foreign DNA fragments. P, priming site of the vector. A*, the end resulting from digestion of A with the restriction enzyme. (a) to (c) Cloning a DNA fragment into the vector. (d) Product of partial digestion of recombinant DNA with DNAase I in the presence of Mn^{2+}. The breaks outside the insert DNA either destroy the phage or remove the priming site (see the text); the breaks within the insert DNA give rise to a mixture of linear double-stranded recombinants with various lengths of insert DNA on both ends. (e) Treatment of linear recombinant DNA with the restriction enzyme and DNA polymerase I Klenow subfragment creates a product with insert DNA pieces at one end and none at the other. (f) Circularization of these linear recombinants with T4 DNA ligase produces a clone mixture with insert DNA fixed at one end and sequentially shortened at the other. Sequencing those clones from the P site covers the whole length of the insert DNA.

3. Materials and Methods

The DNA polymerase I (Klenow subfragment, approx. 1 unit/μl) was obtained from Boehringer Mannheim, and the dNTPs and ddNTPs from P.L. Biochemicals Inc. [α-^{32}P]dATP (spec. act. > 400 Ci/mmol) was from Amersham International Ltd. The primer was made in this laboratory (Duckworth *et al.*, 1981). Restriction enzymes *Sma*I (2 units/μl) and *Hin*dIII (3 units/μl) were from New England Biolabs. DNAase I (grade DN-EP) from Sigma, and low melting agarose from Bethesda Research Laboratories. M13mp9 was originally a gift to Dr F. Sanger from Dr J. Messing. T4 DNA ligase was a gift from D. R. Bentley. The DNA prepared from phage λ *c*I857*S*am7 was from the Centre for Applied Microbiology and Research, Porton.

The lambda DNA used in this study was prepared from phage grown in an r_K^- m_K^+ host strain. JM101 is wild-type for *Eco*K restriction enzyme and methylase; consequently, when it is used as a host cell, DNA fragments containing an *Eco*K site will be cloned very inefficiently unless the DNA to be inserted is methylated at the K site.

(a) *Cloning of* λ Hin*dIII fragment into vector*

The vector used was M13mp9 (J. Messing, unpublished results). It contains the unique restriction enzyme recognition sites in the β-galactosidase gene, as follows:

5' A-A-G-C-T-T-G-G-C-T-G-C-A-G-G-T-C-G-A-C-G-G-A-T-C-C C-C-G-G- G-A-A-T-T-C 3'

*Hin*dIII — *Pst*I — *Sal*I, *Acc*I — *Bam*HI — *Sma*I, *Xma*I — *Eco*RI

The priming site of the vector is a few nucleotides downstream from the *Eco*RI site (Duckworth *et al.*, 1981). The *Sma*I site was used as the restriction site close to the priming region (A site, Fig. 1) and the *Hin*dIII site was used for insertion of the DNA fragments generated by this enzyme (B site, Fig. 1).

1 μg of λ DNA was digested at 37 °C for 2 h in 15 μl H buffer (6·6 mM-Tris·HCl (pH 7·4), 6·6 mM-$MgCl_2$, 6·6 mM-dithiothreitol, 0·05 M-NaCl) containing 3 units of *Hin*dIII. The reaction was stopped by extraction with phenol. The DNA digest was recovered by precipitation with ethanol and dissolved in 20 μl water. 0·5 μl of this DNA solution was cloned (Sanger *et al.*, 1980) using vector M13mp9 in the bacterial host JM101 (a *tra*$^-$ mutant; Messing, 1979). Six *lac*$^-$ ("white") clones were randomly picked and the single-stranded DNA prepared as described by Sanger *et al.* (1980). As all the contiguous readings (Staden, 1980) of the phage λ sequence had already been joined into one consensus (F. Sanger, A. R. Coulson, G. F. Hong, D. Hill & G. B. Petersen, unpublished data), the clones with different *Hin*dIII fragments were easily positioned on the λ genome using ddT reaction screening (Sanger *et al.*, 1980). The size of fragment 1 was confirmed by sequencing in both directions (Sanger *et al.*, 1980; Hong, 1981).

To prepare the replicative form the recombinant was transfected into the bacterial host JM101 by the calcium chloride technique (Cohen *et al.*, 1972). 0·5 l of culture was obtained from growth of a single colony carrying the above recombinant phage. From this 300 μg of the recombinant DNA were isolated by the cleared lysate method (Katz *et al.*, 1973), followed by ethidium bromide/caesium chloride ultracentrifugation (Radloff *et al.*, 1967).

(b) *DNAase I digestion and product isolation*

Samples of DNAase I were stored at −20 °C as a 1 mg/ml solution in 0·01 M-HCl (Rigby *et al.*, 1977) and diluted at least half an hour before use in a digestion buffer composed of 20 mM-Tris·HCl (pH 7·4), 100 μg bovine serum albumin/ml and 1 mM-$MnCl_2$.

12 μg of the replicative form of the recombinant DNA were dissolved in 450 μl freshly prepared DNAase I digestion buffer. The digestion was started at room temperature by mixing with an equal volume of DNAase I at 5 pg/μl in the same digestion buffer. The digestion mixture was divided into 3 equal portions. One was digested for 5 min and the digestion stopped by extraction with an equal volume of phenol. The other 2 were digested

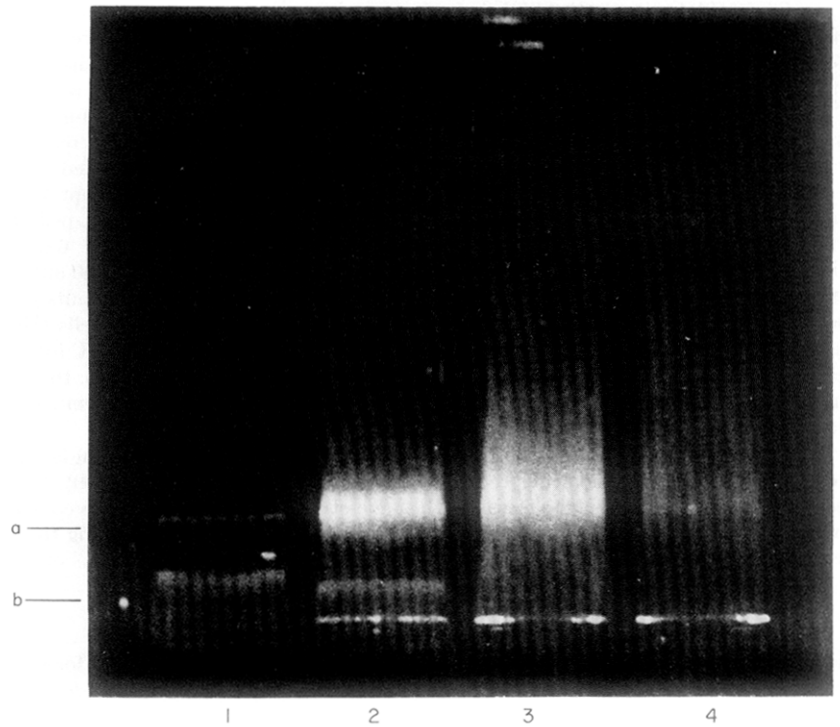

FIG. 2. Electrophoresis of DNAase I digest of the recombinant DNA on a 1% low-melting agarose gel. in Tris-borate buffer (pH 8·6). 1 The replicative form of the recombinant DNA with 2327 base-pair *Hin*dIII fragment and its linear form, produced by digestion with *Sma*I. a, Indicates the position of the linear form; b, the position of the replicative form. 2, 3 and 4 show the DNAase I digestion of the recombinant DNA at room temperature for 5, 10 and 15 min, respectively. The disappearance of band b in tracks 3 and 4 implies over-digestion. Band a in track 2 was extracted and the DNA used to make a clone mixture (see the text).

for 10 and 15 min and the digestion stopped in the same way. The DNA was recovered from each portion by precipitation with ethanol and dissolved in 10 μl water, mixed with 6 μl of 40% (w/v) sucrose and 0·2% (w/v) bromophenol blue and applied to a 1% (w/v) low melting agarose gel for electrophoresis (Fig. 2). The DNA was stained with ethidium bromide. There were 2 bands on the track where the 5 min digestion mixture was loaded. The faster moving band, which corresponded to the linear double-stranded form of the recombinant, was cut out, the agarose melted at 70°C and extracted with phenol. The recovered DNA was dissolved in 16 μl water.

(c) Sma*I digestion and transfection*

16 μl of the above DNA was mixed with 2 μl 10 × H buffer and 2 μl *Sma*I (2 units/μl), and the mixture incubated at 37°C for 2 h. The digestion was stopped by heating at 70°C for 10 min. To this were added 1 μl 10 × H buffer, 7 μl of a mixture containing the 4 dNTPs at 0·125 mM concentration and 2 μl DNA polymerase I (Klenow subfragment) (1 unit/μl). The mixture was left at room temperature for 30 min and then the enzyme inactivated in the same way.

30 μl 10% polyethylene glycol-6000 containing 1·2 M-NaCl was added to the above mixture. The polyethylene glycol mixture was left on ice for 1·5 h (or at 4°C overnight) and then centrifuged in an Eppendorf centrifuge for 10 min. The supernatant was discarded, the last drop being removed with a drawn-out capillary tube. The precipitate was washed with 60 μl 5% polyethylene glycol containing 0·6 M-NaCl. After 5 min centrifugation the supernatant was again removed in the same manner. The DNA precipitate was resuspended in 50 μl 10 mM-Tris·HCl (pH 7·4) and 0·1 mM-EDTA, and this solution extracted once with 30 μl phenol. 5 μl 5 M-NaCl and 150 μl ethanol were added and the mixture kept at −20°C overnight or in a solid CO_2/ethanol mixture for 20 min. The DNA was pelleted by centrifugation, washed once with ethanol and then dissolved in 10 μl water. 6 μl of this solution was mixed with 2 μl 5 × ligase buffer (250 mM-Tris·HCl (pH 7·5), 50 mM-$MgCl_2$, 25 mM-dithiothreitol, 1 mM-rATP), 1 μl water and 1 μl T4 DNA ligase (10 units/μl). The ligation mixture was incubated at 15°C overnight. 0·2 ml of the competent cells (Messing, 1979) was mixed with the above ligation mixture; it was allowed to stand at 0°C for 40 min, heated at 45°C for 2 min and then plated out on one H plate (Sanger *et al.*, 1980). 160 plaques of various sizes were obtained. No isopropyl-β-D-thiogalactopyranoside or 5-bromo-4-chloro-3-indoyl-β-galactoside were necessary in this step.

24 plaques of various sizes were picked from the agarose plate and the single-stranded cloned DNA was prepared using the procedure as described by Sanger *et al.* (1980). The DNA samples were subjected to electrophoresis with single-stranded M13mp9 DNA as control on a 1% (w/v) agarose gel. Four DNA samples ran faster than the control, indicating that DNA stretches longer than insert DNA had been deleted in these clones ("nonsense" clones).

(d) *ddT reaction screening*

One of the 4 dideoxynucleotide termination sequencing reactions can be used for screening clones. Here the ddT reaction screening was performed using the conditions described by Sanger *et al.* (1980) except that both 6% and 12% sequencing gels were used for screening all except the 4 nonsense clones: the 6% gel was run at 30 mA for 4·5 h and the 12% gel at 30 mA for 1 h using 40 cm × 20 cm thin sequencing gels (Sanger & Coulson, 1978).

4. Results

(a) *Monitoring and isolation of the DNAase I digest*

The extent of DNAase I digestion was easily controlled by using the same concentration with varying periods of digestion time (Fig. 2). Where incubation was for longer than 10 minutes over-digestion was observed. In Figure 2 band a corresponds to the linear double-stranded form of the original recombinant and band b to its replicative form. Band b on track 2 was found to be a useful signal showing the suitable extent of digestion. Under the separation conditions described in this paper the disappearance of this band indicated over-digestion; i.e. the number of surviving phages and the recombinants with longer insert DNA were greatly reduced.

The linear double-stranded form (band a), as exhibited on the agarose gel (Fig. 2), indicated that basically one hit had occurred randomly for each molecule. It was necessary to separate the linear form of the recombinants (band a) from the replicative form on low melting agarose gel for transfection. Without this separation about 40% of the total recombinants prepared afterwards were found to be the original one.

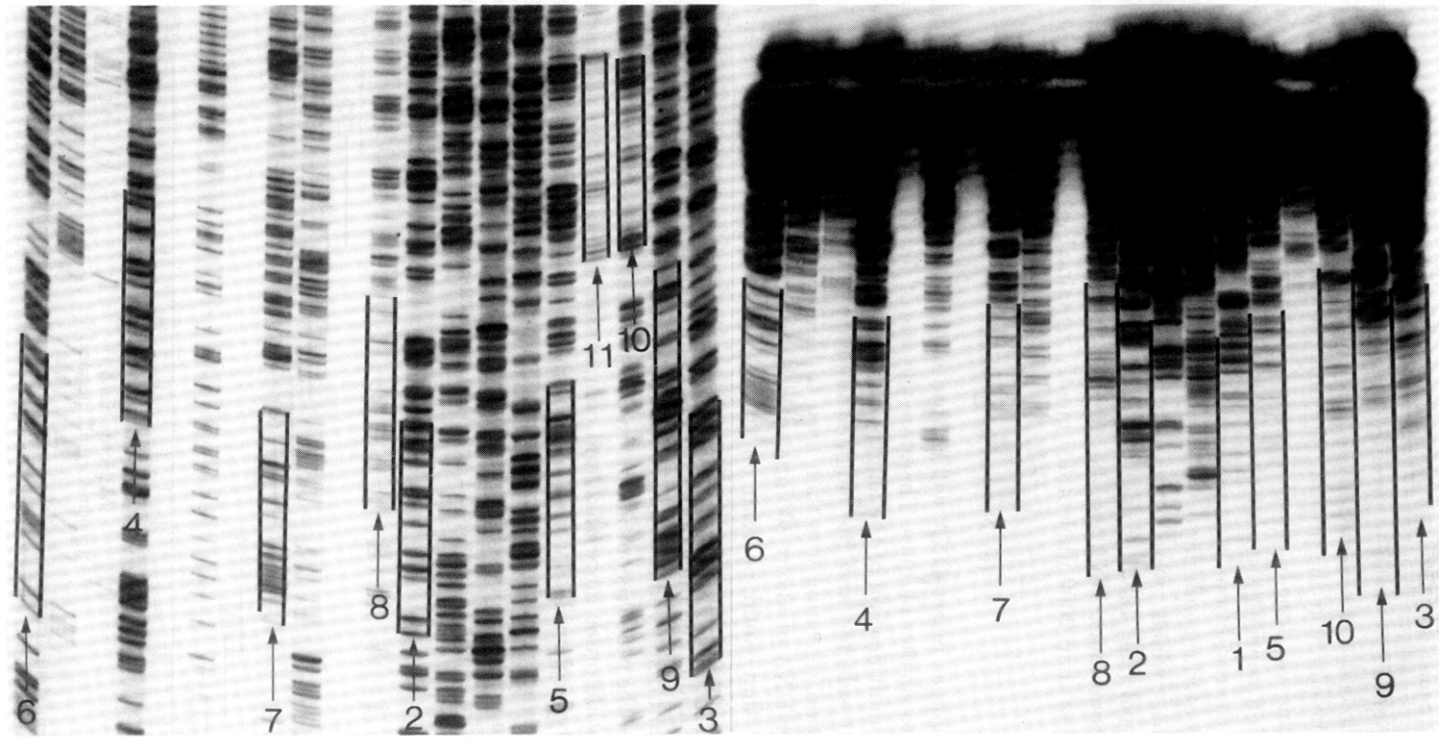

FIG. 3. ddT reaction screen patterns of all single-stranded phage DNAs except 4 "nonsense" clones (see the text). (a) Autoradiograph of 6% polyacrylamide/8 M-urea DNA sequencing gel run for 4·5 h; (b) autoradiograph of a 12% sequencing gel run for 1 h.

(b) Sma*I digestion and polyethylene glycol precipitation*

As shown in Figure 1, in order to produce a recombinant mixture with the insert DNA fixed at one end and sequentially degraded at the other, all the insert DNA pieces stuck to the end near the priming site must be removed after digestion with DNAase I, and this was done by *Sma*I digestion. As complete digestion is necessary at this step a high concentration of the enzyme and a long digestion time were required. Under the conditions used no wrong ligations were detected. Digestion of the DNAase I-treated recombinants with *Sma*I created a mixture of DNA pieces in different lengths, which were efficiently removed by precipitation with polyethylene glycol (Lis, 1980). Under the conditions described in Materials and Methods the recombinant DNA can be precipitated almost quantitatively, and DNA pieces of less than 2000 nucleotides mostly remained in the supernatant.

(c) *Screening of clones*

Viruses with longer insert DNA will diffuse more slowly on the agarose plate, thus giving rise to smaller plaques. Therefore the size of the plaques is a rough, but useful, guide to identification of the length of insert DNA. A total of 24 plaques of various size were selected and the single-stranded DNA prepared (Sanger *et al.*, 1980). ddT reaction screening was then used to identify the suitable recombinant containing insert DNA of about 200 nucleotides difference in length.

The sequence that could be read on the 6% gel was between 120 and 300 nucleotides from the priming site. If there was an overlapping region between two recombinants of about 200 nucleotides difference in length, the nucleotide pattern of one recombinant displayed on a longer run sequencing gel must be exhibited on a shorter run gel of the other recombinant. In order to make these two patterns with the same nucleotide order more alike and comparable the longer run was carried out on a 6% polyacrylamide/8 M-urea sequencing gel for 4·5 hours and the shorter run on a 12% polyacrylamide/8 M-urea gel for 1 hour (see Materials and Methods). The ddT reaction mixtures of each recombinant were run on both 6% and 12% gels. As shown in Figure 3, very similar ddT reaction patterns were displayed on 6% and

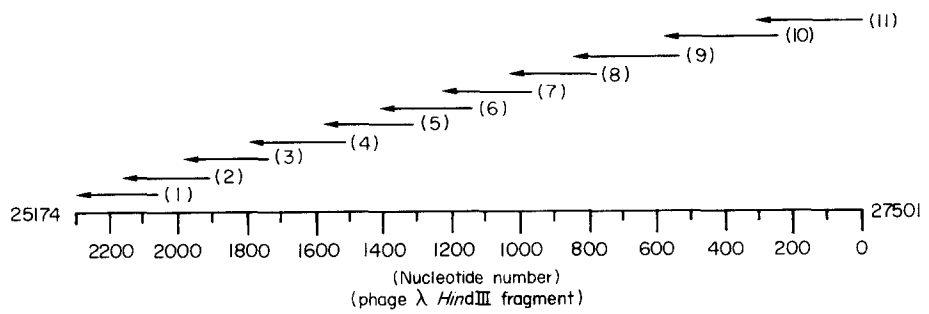

FIG. 4. Distribution along fragment 1 of the sequencing region of the individual clones used in this work. (1) to (11). the individual clones as shown in Fig. 3; arrows indicate sequencing direction.

12% gels for the recombinants having overlapping regions. Track 11 of the 6% gel displays the pattern of the clone not digested by DNAase I. This pattern was found on track 10 of the 12% gel, indicating overlapping between those two clones. Following the same rule, track 10 on the 6% gel shows overlapping with track 9 on the 12% gel, track 9 with track 8, and so on. Therefore all the clones were found that had about 200 base-pairs difference in length among them. The whole length of the insert DNA was thus covered by them. (For clone distribution see Figure 4. For overlapping region and DNA sequence of fragment 1 see Figure 5.) Several blank ddT tracks can be seen in the ddT screening patterns, indicating deletion of the priming sites of these recombinants.

5. Discussion

This paper describes a progressive DNA sequencing strategy based on the use of the dideoxynucleotide chain termination sequencing system and single-stranded phage vector derivatives. This circumvents the inherently asymptotic nature of sequence accumulation in the "shotgun" method. The length of DNA along which the sequence can be read progressively appears to be limited only by the insertion capacity of the vector. Recombinants with insert DNA ranging from several hundreds to about 6500 nucleotides from a digest of phage λ DNA have been obtained in this way and have remained stable. Combined with the physical map, this method has considerable potential for the progressive sequencing of a whole genome. Where the shotgun method has been employed initially, it can be combined with this method to confirm specific regions or to select specific recombinants to fill in the final few gaps. If the cloned sequence possesses blocks or regions of repeated sequence, this might interfere with the logical development of the overlapping.

As can be seen from Figure 1, after treatment with DNAase I and *Sma*I, the main requirement for the progressive sequencing technique is the selection of recombinant DNA of about 200 nucleotides difference in length. One way to identify suitable recombinants is by sizing the insert DNA. Several fast and simple methods have been available to prepare the replicative form of recombinant DNA (Holmes & Quigley, 1981; Birnboim & Doly, 1979). The recombinant DNA so prepared was digested with appropriate restriction enzymes to release the insert DNA, which was then subjected to electrophoresis on a 1% agarose gel to determine the length of the inserts. This procedure, as well as needing more unique restriction enzyme recognition sites, is much less accurate than the screening procedure, especially with recombinants where the insert DNA is longer than 1000 nucleotides. The ddT reaction procedure, on the other hand, is size-independent and very accurate. Furthermore, the screening patterns give overlapping regions between two or more recombinants, which allows the selection of more suitable individual recombinants to cover the whole insert DNA. When the whole insert DNA has been covered by the recombinants selected, the ddT patterns of other recombinants can still be useful for confirming specific regions.

As shown in Figure 1, at least two unique restriction enzyme recognition sites were needed for this strategy. A restriction site (flush end or sticky end with a

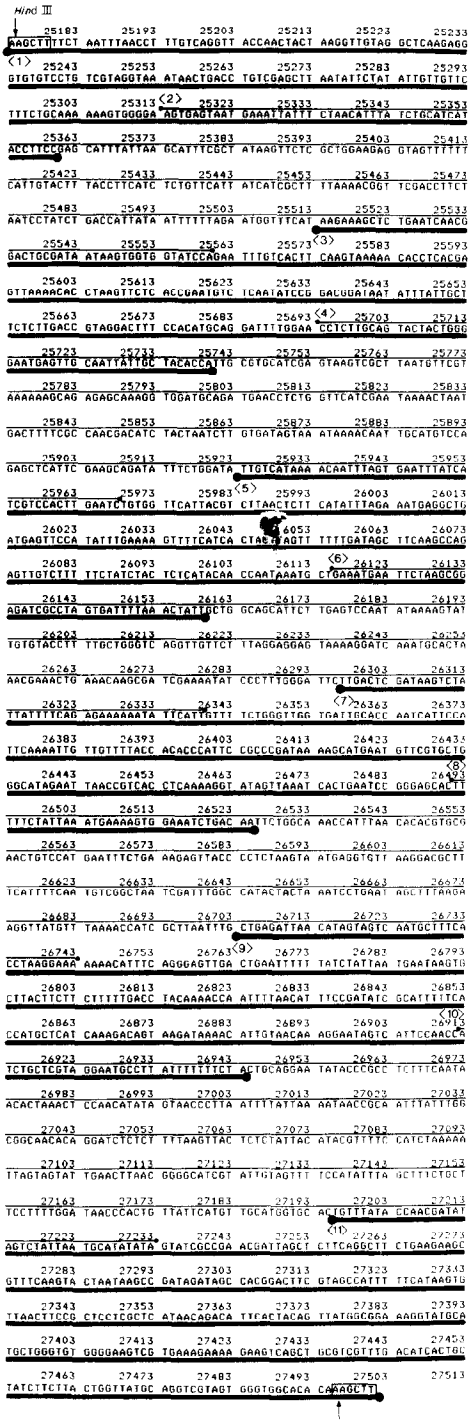

FIG. 5. Nucleotide sequence of fragment 1. The numbers ⟨1⟩ to ⟨11⟩ refer to individual gel readings. indicated by lines above or below the sequence. Sequences between 2 lines are overlapping regions composed of 33 to 48 base-pairs. Hyphens have been omitted from the sequence for clarity.

recessed 3′ end) close to the priming site of the vector was required for the progressive shortening of insert DNA: this site should be as infrequent as possible to limit the chance of breakage occurring within the insert DNA. The other restriction site is used for cloning DNA fragments and it can be either sticky or flush end. A fragment of about 1600 nucleotides from pBR322 has been inserted into another M13 derivative with flush ends without difficulty when the concentrations of both T4 DNA ligase and the fragment to be inserted were enhanced in the ligation reaction.

In this paper the cloned DNA was sequenced in one orientation. Where a sequence is not known it is usually necessary to sequence in both directions. If a clone with the insert in the opposite orientation is not available, it can be obtained simply by recutting and religating the original clone (Winter & Fields, 1980).

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