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PRINCIPAL INVESTIGATOR: Sidney M. Hecht, Ph.D.

CONTRACTING ORGANIZATION: University of Virginia  
School of Medicine  
P.O. Box 9003  
Charlottesville, VA 22906

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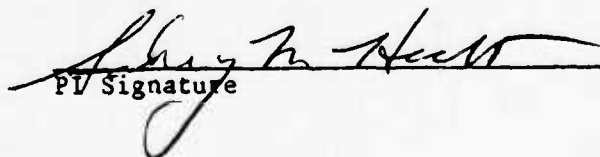
  
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## INTRODUCTION

We have been carrying out a systematic search for natural products that may act as antimalarial agents by virtue of their ability to inhibit *Plasmodium falciparum* DNA polymerase  $\alpha$  (ie, the aphidicolin-sensitive polymerase activity), an enzyme believed to be absolutely essential to the viability and function of the parasite. Because isolation of the enzyme requires very large amounts of *Plasmodium*, relative to what is obtained routinely from infected cells, we have pursued a parallel strategy in which we are attempting to clone the enzyme, while separately identifying extracts likely to have at least some activity as inhibitors of the malarial polymerase  $\alpha$ .

## BODY

As outlined in our original grant proposal, earlier work screening for inhibitors of trypanosome DNA polymerase  $\alpha$  demonstrated that a number of extracts contained potent inhibitors of that enzyme which were much less potent as inhibitors of DNA polymerases  $\alpha$  and  $\beta$  derived from a human cell line (Table I). As anticipated, these extracts actually did inhibit thymidine and leucine uptake/utilization by cultured trypanosomes (Table II).

In the belief that the same principles can be utilized to find selective, potent inhibitors of *Plasmodium falciparum* DNA polymerase  $\alpha$  that can act as antimalarial agents, we have undertaken an analogous search in that system. At a conceptual level, the search for selective inhibitors of *Plasmodium falciparum* DNA polymerase  $\alpha$  may be envisioned as follows:

1. Purify and characterize *P. falciparum* DNA polymerase  $\alpha$
2. Identify plant extracts having potent, selective inhibitory activity toward the malarial DNA polymerase  $\alpha$ , but not toward human (i.e., KB cell) DNA polymerases  $\alpha$  or  $\beta$
3. Fractionate several extracts containing the putative inhibitors and obtain the active principles by bioassay-guided fractionation
4. Elucidate the chemical structures of the isolated principles
5. Obtain the active principles in quantities sufficient for *in vitro* and *in vivo* antimalarial testing by WRAIR in experimental animal models.

As a practical matter, however, we cannot pursue this approach at present because we do not have any *P. falciparum* DNA polymerase  $\alpha$ . Therefore, we are pursuing a modified strategy in which we carry out three types of activities in parallel. These are

1. Identify potential inhibitors of malarial DNA polymerase  $\alpha$  by the use of polymerases from other organisms (*vide infra*)
2. Attempt to secure an unlimited supply of the *P. falciparum* DNA polymerase  $\alpha$  by cloning the gene for the enzyme

3. Prepare additional plant extracts to support our ongoing screening efforts.

Presently, we are using a modified scheme to identify putative inhibitors of *Plasmodium falciparum* DNA polymerase  $\alpha$  that does not require the actual availability of any of the malarial enzyme. This is

1. Identify plant extracts weakly or moderately inhibitory toward human DNA polymerase  $\alpha$
2. Dereplicate extracts that utilize phenolic compounds as DNA polymerase  $\alpha$  inhibitors
3. Test the confirmed active extracts as inhibitors of cultured *P. falciparum* and as inhibitors of the growth of human cells
4. Fractionate extracts that
  - inhibit human DNA polymerase  $\alpha$
  - inhibit cultured *P. falciparum*
  - do not inhibit the growth of cultured Vero cellsto isolate the active principles by bioassay (*P. falciparum* inhibition, DNA polymerase  $\alpha$  inhibition)-guided fractionation
5. Identify active principles and characterize them as antimalarial agents

The logic of this scheme rests on the observation (Table I) that strong inhibitors of DNA polymerase from a given organism (eg, trypanosomes) will generally have at least some activity as inhibitors of the polymerases from other organisms (eg, human DNA polymerases  $\alpha$  and  $\beta$  in Table I). While it obviously does not follow that extracts found to be weakly or moderately inhibitory towards, eg human DNA polymerase  $\alpha$  will necessary be strongly inhibitory toward trypanosomal (or malarial) DNA polymerase  $\alpha$ , the set of extracts that has some activity against human DNA polymerase  $\alpha$  probably does include most of those extracts that would be found to be strong inhibitors of the malarial polymerase  $\alpha$  were the *P. falciparum* enzyme available for direct assay.

Therefore, we employed a commercially available sample of human (KB cell) DNA polymerase  $\alpha$  (from Molecular Biology Resources, Inc.,



Milwaukee, Wisconsin) to screen a number of plant extracts. The assay system consisted of the enzyme-mediated incorporation of [<sup>3</sup>H]dATP into an oligo(A)<sub>12-18</sub> primer into the presence of poly(dT) template. Each of 343 extracts was tested as an inhibitor of [<sup>3</sup>A]dATP incorporation at no fewer than two concentrations. Because we know from experience that polyphenols often mimic the (polyanionic) nucleic acids, and thereby give non-specific inhibition of nucleic acid-utilizing enzymes, we dereplicated phenolic compounds by retesting the inhibitory extracts in the presence of varying concentrations of bovine serum albumin (a basic protein that binds tightly to most polyphenols). On the basis of these (728) assays, we chose a number of extracts that were moderate inhibitors of human DNA polymerase  $\alpha$  (Table III), and which retained some or all of their inhibitory activity in the presence of bovine serum albumin (Table IV).

The extracts so identified were tested as inhibitors of two clones of cultured *Plasmodium falciparum*. As shown in Table V, four of these extracts (marked by asterisks in the Table) were reasonably good inhibitors of the cultured organisms, especially when one considers that typically only 0.10-1.0% of the extract consists of the active principle producing the observed inhibitory effect. On the assumption that the abundance of the putative antimalarial agents in the extracts is typical, the potency of the agents must compare favorably with those of the authentic antimalarial agents shown in the Table.

The same extracts were tested for cytotoxicity toward cultured Vero cells to determine whether the putative antimalarial principles would be less toxic toward mammalian cells. As illustrated in Table VI, none of the extracts was strongly cytotoxic toward Vero cells.

We believe that the four extracts identified in Table V represent logical candidates for fractionation, guided both by inhibitory activity toward KB cell DNA polymerase  $\alpha$  and toward cultured *Plasmodium falciparum*. We intend to initiate fractionation of one or more of these extracts soon, although we may delay the choice of extract briefly if there is a reasonable chance of obtaining a sample of the cloned or *P. falciparum*-derived DNA polymerase  $\alpha$  in the near future (*vide infra*).

As noted above, given the difficulty of obtaining sufficient *P. falciparum* to carry out enzyme isolation, the only realistic method for obtaining routine supplies of DNA polymerase  $\alpha$  from this organism is cloning the gene and expressing it in an appropriate host. This is being carried out in collaboration with our subcontractor, SmithKline Beecham Pharmaceuticals.

Very little is known about the replicative DNA polymerase of

*Plasmodium falciparum*. The enzyme has not been purified and characterized, nor has the gene been sequenced. We decided to begin our cloning effort by capitalizing on available information concerning apparently conserved areas of amino acid sequence in other DNA polymerases (Wong et al., 1988), Figure 1 illustrates the observation that 4 or 6 conserved regions appear in several polymerases (or similar proteins) and that the relative order of these regions also appear to be conserved (Wang et al., 1989). As summarized in the Experimental Cloning Strategy, we decided that DNA primers could be synthesized that would be complimentary to conserved regions II and I: these primers could then be used to amplify DNA between the two primer sites using polymerase chain reaction (PCR). The resulting DNA would then be used as probes for a genomic library. Success in identifying sequences in the genomic library would then give adequate justification, and additional probes, for pursuing full length polymerase genes from a cDNA library for subsequence expression.

The high degree of homology that exists between human and yeast DNA polymerase  $\alpha$  (Wang et al., 1989) led us to decide to construct primers that were a synthesis of the two sequences. Where there were differences in sequence we opted for adenine or thymine bases since *falciparum* DNA is generally AT rich. Using the sequence information shown in Figure 2, we constructed the primers shown in Figure 3. Genomic DNA (obtained from David Lanar of WRAMC) was used as the source of *P. falciparum* sequence which was then amplified using our primers as described in Scheme I. The PCR step was successful and we obtained a DNA product about 200 bp long.

Amplification of *P. falciparum* DNA with these "consensus" primers has the obvious advantage of generating probes the majority of which have authentic *P. falciparum* sequence and have sufficient avidity as to survive PCR temperature cycling. However, we also have been pursuing two parallel approaches: (1) primers with authentic yeast sequence was used to amplify yeast DNA using PCR, and (2) the analogous process with human material. We have obtained PCR product from yeast with a size of about 550 bp. The approach has not yet been successful with human DNA.

The PCR product was inserted into the pUC18 vector (see Figure 4) and cloned. Several individual colonies were isolated and the inserts of eight were sequenced. We found that of these eight, six inserts had the same sequence (#1) and the other two had a second sequence (#7). Translation of DNA sequences from these inserts revealed a very slight amount of amino acid homology with the conserved regions described by Wong et al. (1988). While the lack of strong homology is disappointing, it does not indicate that these DNA fragments will fail to serve as probes for obtaining polymerase gene(s) from *P. falciparum* DNA.

A genomic library had been constructed from *P. falciparum* DNA by cutting with restriction endonucleases and inserting the pieces into  $\lambda$ gt11 bacteriophage vector (summarized in Figure 5). We end-labeled our PCR products with [<sup>32</sup>P]phosphate and probed the genomic library using clonal dilution and southern blotting as summarized in Figure 6. Probing the library with *P. falciparum* probe #1 yielded four clones, all of which contained inserts of the same size. Probing with *P. falciparum* probe #7 yielded five clones the inserts in which were different sizes. Probing with yeast polymerase sequence yielded three clones that have not yet been purified.

Inserts obtained from phage  $\lambda$ gt11 were inserted into pUC18 plasmids for sequencing. The status of that effort is still early and is summarized in Figure 7.

In addition to this effort to clone the gene for *P. falciparum* DNA polymerase  $\alpha$ , we have discussed with Major Wilbur Milhous the possible preparation of *P. falciparum* in quantities (2-4 g) sufficient to allow us to carry out an enzyme preparation. This would greatly facilitate the choice of extracts for fractionation (*vide supra*), and will also be necessary in due course for comparison with the enzyme expressed following our gene cloning experiments.

#### CONCLUSIONS

Three parallel efforts are in progress to

- obtain aphidicolin-sensitive *P. falciparum* DNA polymerase  $\alpha$  in quantities sufficient for screening, and to guide the fractionation of extracts containing potent and selective enzyme inhibitors
- select extracts that contain putative inhibitors of DNA polymerase  $\alpha$  that inhibit cultured *Plasmodium falciparum*
- prepare additional extracts for screening to maximize the prospects for finding a potent DNA polymerase  $\alpha$  inhibitor

All three areas have yielded steady progress, and we believe that we can meet the goals of this project.

#### REFERENCES

Wong, S.W., Wahl, A. F., Yuan, P.-M., Arai, N. Pearson, B. E., Arai, K.-I. Korn, D., Hunkapiller, M. W., and Wang, T. S.-F. (1988) **EMBO J. 7**, 37-47.

Wang, T. S.-F., Wong, S. W. and Korn, D. (1989) **Fed. Am. Soc. Exp. Biol. J. 3**, 14-21.

Table I. Assay of Plant Extracts as Inhibitors of Trypanosome DNA Polymerase  $\alpha$ , and Human DNA Polymerases  $\alpha$  and  $\beta$ .

Extract (10 $\mu$ g/mL)	% Inhibition		
	Trypanosome DNA Polymerase $\alpha$	Human DNA Polymerase $\alpha$	Human DNA Polymerase $\beta$
B855435-5	86.8	37.2	91.6
B855434-5	97.1	42.0	65.7
B855407-5	97.6	51.9	15.9
B855394-5	95.5	58.3	37.0
BRS-2-146-5	97.0	13.0	57.4
PC-9-145-5	94.6	13.5	8.1
PC-7-12-5	94.0	0.4	27.6
BRS-3-190-5	94.9	23.3	12.1
B855892-5	93.1	0.6	74.3
B855307-5	92.1	35.7	8.4
Aphidicolin	-----	70.8	-----

Table II. Effect of Plant Extracts Containing DNA Polymerase  $\alpha$  Inhibitors on Thymidine and Leucine Uptake/Utilization by Cultured *Trypanosoma rhodesiensis*.

Extract	Thymidine (IC-50; $\mu\text{g}/\text{mL}$ )	Leucine (IC-50; $\mu\text{g}/\text{mL}$ )
B855435-5	9.7	9.7
B855434-5	2.4	9.7
B855407-5	3.0	9.6
B855394-5	19.5	39.0
BRS-2-146-5	---	---
PC-9-145-5	2.6	41.4
PC-7-12-5	61.7	---
BRS-3-190-5	5.0	10.5
B855892-5	40.0	---
B855307-5	1.72	7.28
ethidium bromide	0.6	2.43

Table III. Testing of Plant Extracts as Inhibitors of  
Human DNA Polymerase  $\alpha$

<u>Extract</u>	<u>Extract Concentration (<math>\mu\text{g}/50 \mu\text{L}</math>)</u>	<u>% Inhibition</u>
B856371-7 (7)*	0.2	36
B855484-7 (34)	0.2	37
B855485-7 (11)	0.2	78
B856368-7 (8)	0.2	37
B855448-7 (6)	0.2	51
B856331-7 (11)	0.4	45
B855362-7 (3)	0.2	36
B856382-7 (3)	0.2	35
B856384-7 (5)	0.4	49
B855346-7 (7)	0.2	68
B856381-7 (5)	0.2	60

\* Number of assays

Table IV. Effect of Bovine Serum Albumin on the Inhibition of Human DNA Polymerase  $\alpha$  by Plant Extracts

Extract	BSA ( $\mu\text{g/mL}$ )	% Inhibition
B855484-7 (0.31 unit of enzyme)	0	74
	10	48
	25	23
	50	13
	75	34
B855484-7 (1 unit of enzyme)	10	46
	25	37
	50	26
	75	31
B855485-7	0	94
	100	78
B855448-7	0	88
	50	51
B855362-7	0	83
	50	36



Table V. Testing of Plant Extracts as Inhibitors of Cultured Plasmodium falciparum

Extract	IC <sub>50</sub> (µg/mL)	
	D6 African Clone	W <sub>2</sub> Indochina Clone
* B856371-7	3.36	~10
B855484-7	104	87.5
B855485-7	60.9	110
* B856368-7	7.9	32
B855448-7	22.8	102
* B856331-7	14.2	11.8
B855362-7	109	~100
B856382-7	208	205
B856384-7	202	198
* B855346-7	30.8	19.1
B856381-7	89.2	29.7
mefloquine	4.05 x 10 <sup>-3</sup> †	1.75 x 10 <sup>-3</sup>
chloroquine	1.2 x 10 <sup>-3</sup>	7.47 x 10 <sup>-3</sup> †
quinine	4.97 x 10 <sup>-3</sup>	37.7 x 10 <sup>-3</sup>
artemisinin	1.96 x 10 <sup>-3</sup>	0.72 x 10 <sup>-3</sup>
arteether	0.93 x 10 <sup>-3</sup>	0.66 x 10 <sup>-3</sup>

† Atypically low IC<sub>50</sub> value

Table VI. Testing of Plant Extracts as Inhibitors of Cultured Vero Cells

Extract	Vero Cell IC <sub>50</sub> ( $\mu\text{g/ml}$ )
B855346-7	>>20 <sup>a</sup>
B855362-7	20
B855448-7	>>20
B855484-7	>20 <sup>b</sup>
B855485-7	>20
B856331-7	>>20
B856368-7	>>20
B856371-7	18
B856381-7	>20
B856382-7	>>20
B856384-7	>20

a No inhibition at 20  $\mu\text{g/ml}$  indicated by >>20

b 20-30 percent inhibition at 20  $\mu\text{g/ml}$  indicated by >20

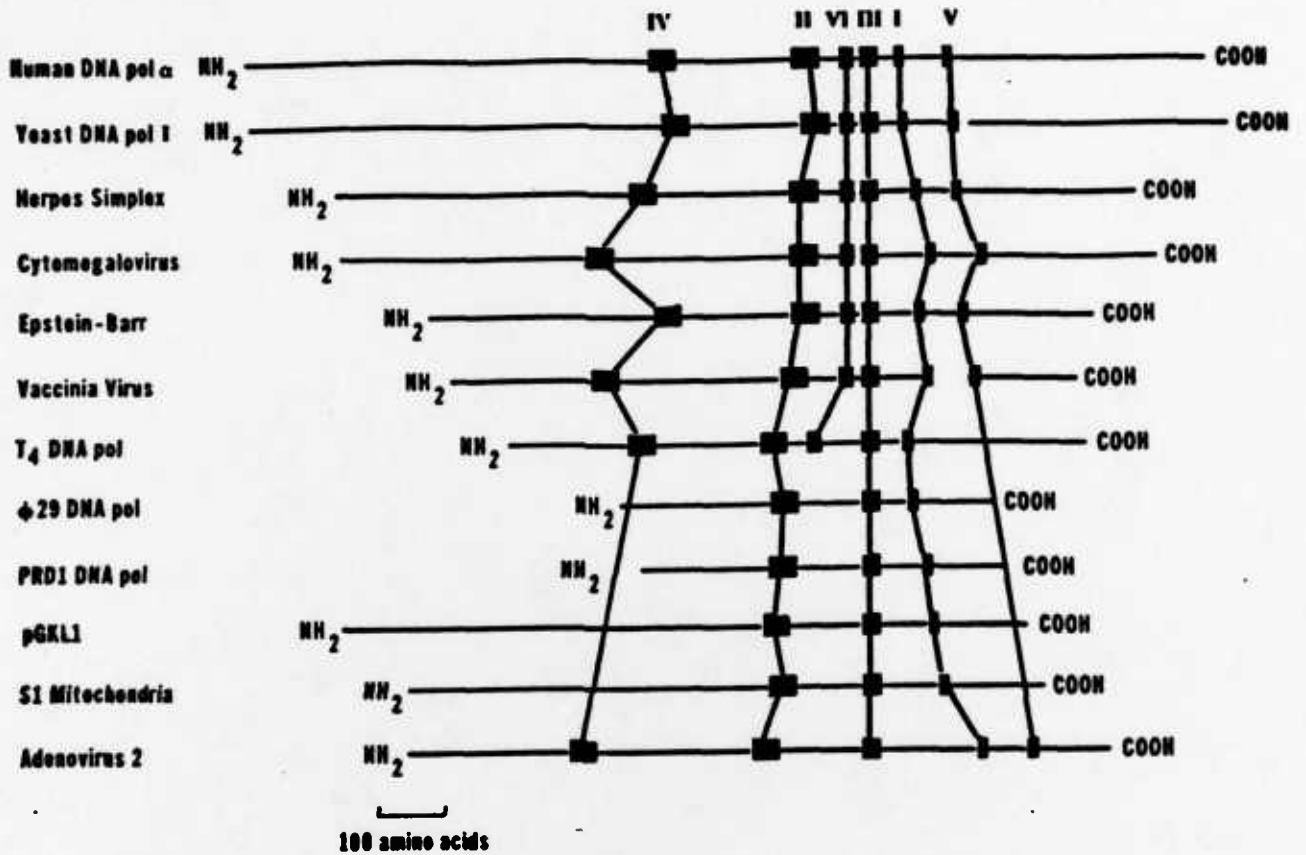


Fig. 1. Conserved Regions among DNA Polymerases.

## Experimental Strategy

1. **Design oligonucleotide primers to consensus regions I and II of *pol* gene using similarities between yeast and human sequences. Also, prepare primers specific for the known sequences of yeast and human *pol* genes.**
2. **Use PCR with *falciparum* DNA and oligo primers to amplify a small region of *falciparum pol* DNA. Also, use yeast and human primers to amplify the corresponding section of their respective DNA.**
3. **Insert amplified DNA into the pUC18 vector and determine its sequence.**
4. **Screen bacteriophage  $\lambda$ GT11 *falciparum* genomic library using PCR product as a probe to pick out *falciparum* polymerase gene.**
5. **Obtain/prepare a *falciparum* cDNA library and screen for full-length *pol* DNA that can be placed into an appropriate expression system.**
6. **Purify and characterize recombinant DNA polymerase.**

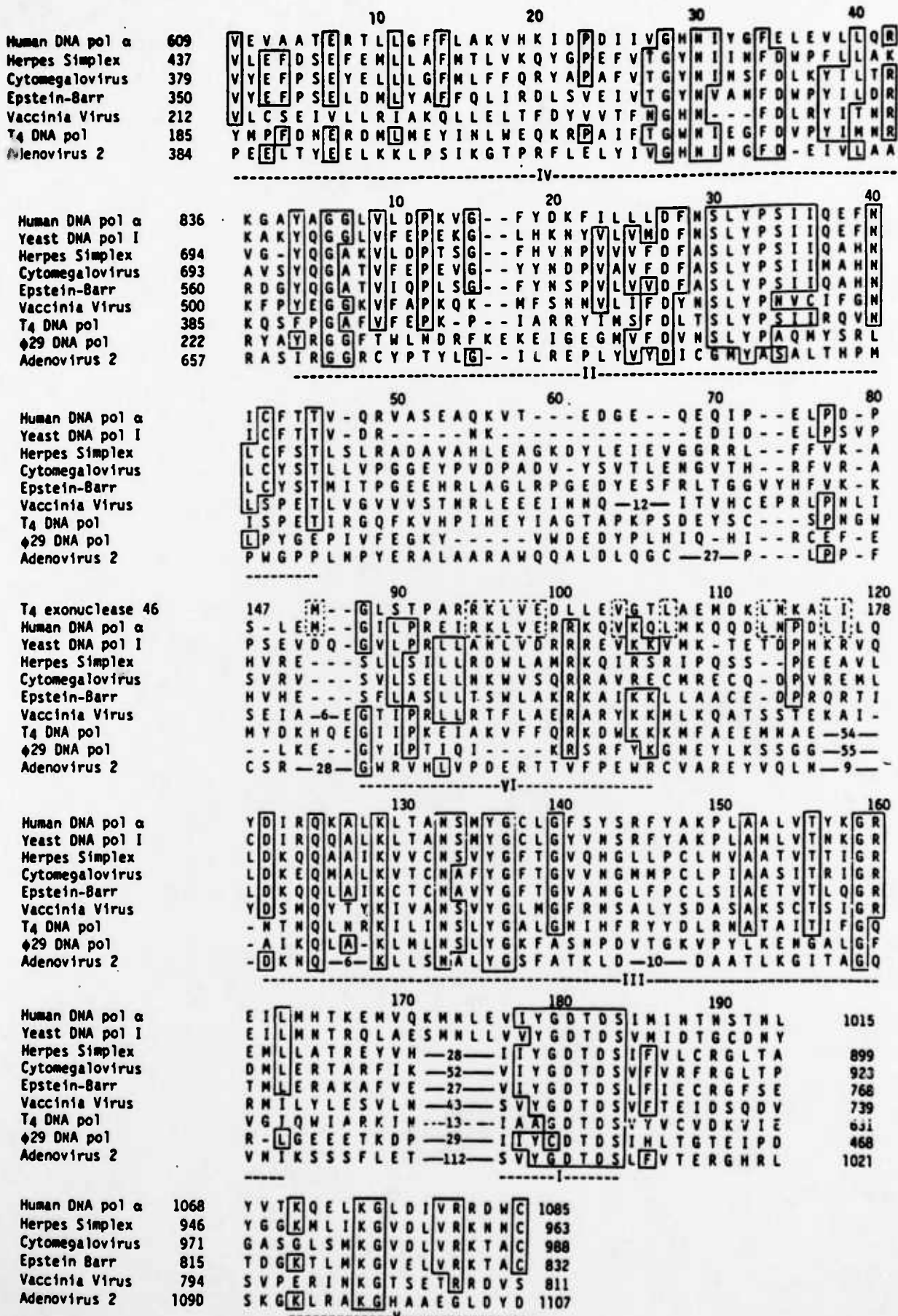


Fig. 2. Sequences of DNA Polymerases from a number of sources

consensus region I antisense primer (40-mer):

5' CAGTGGATCCCCATAACTGAATCTGTATCTCCATAATTAC 3'

consensus region II sense primer (40-mer):

5' CAGTCTCGAGATTATTCAGGAATTTAATATTTGTTTTACA 3'

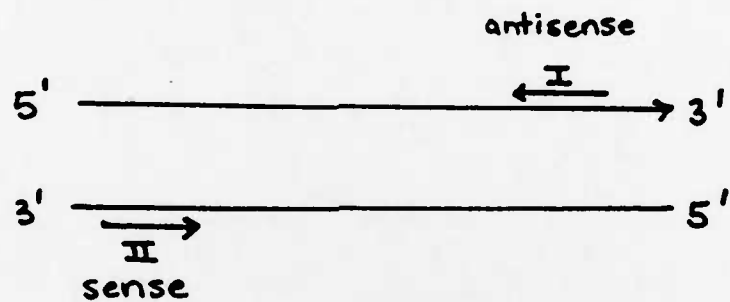
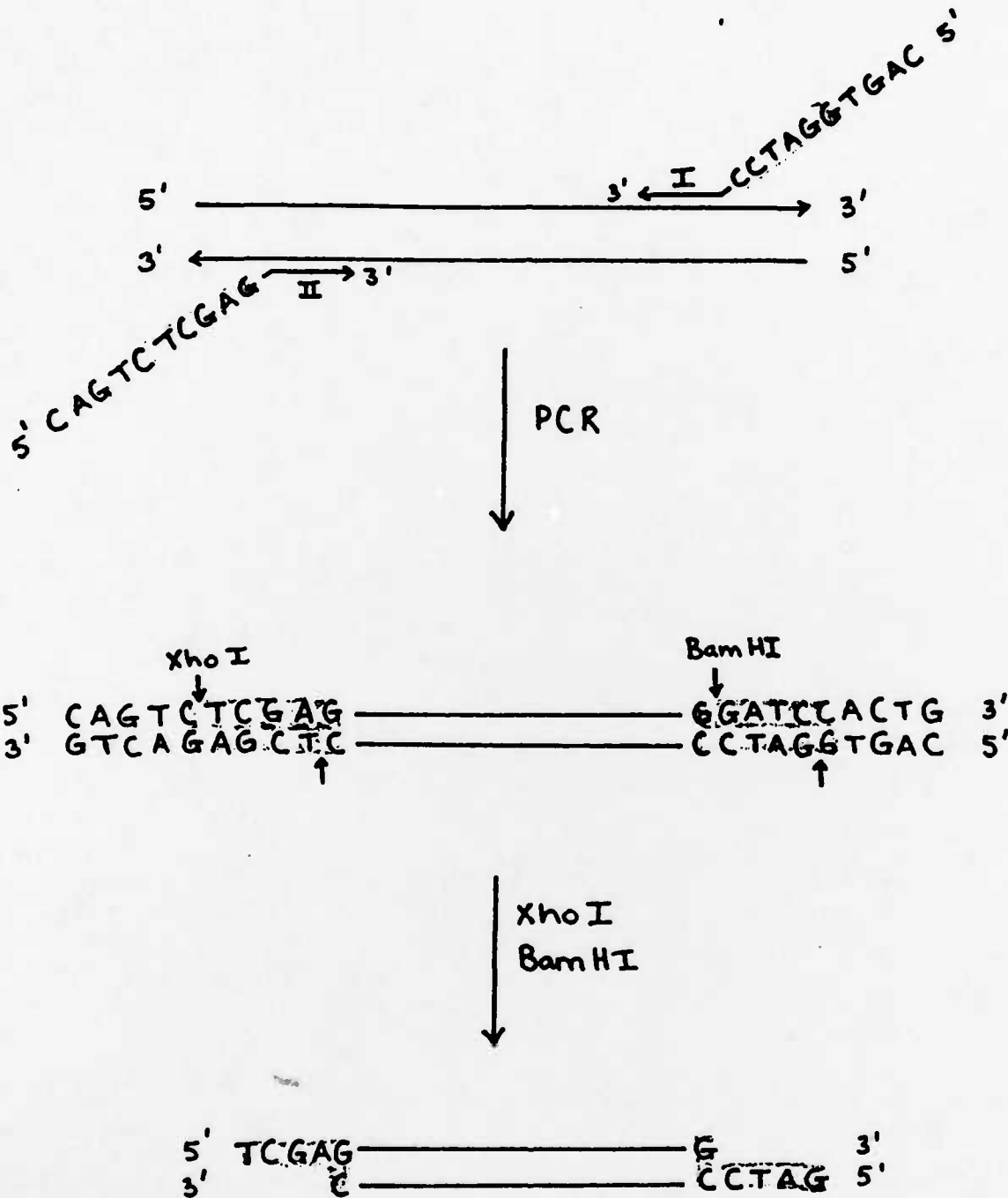


Fig. 3. P. falciparum DNA polymerase consensus primers.



Scheme I. PCR-mediated primer amplification.

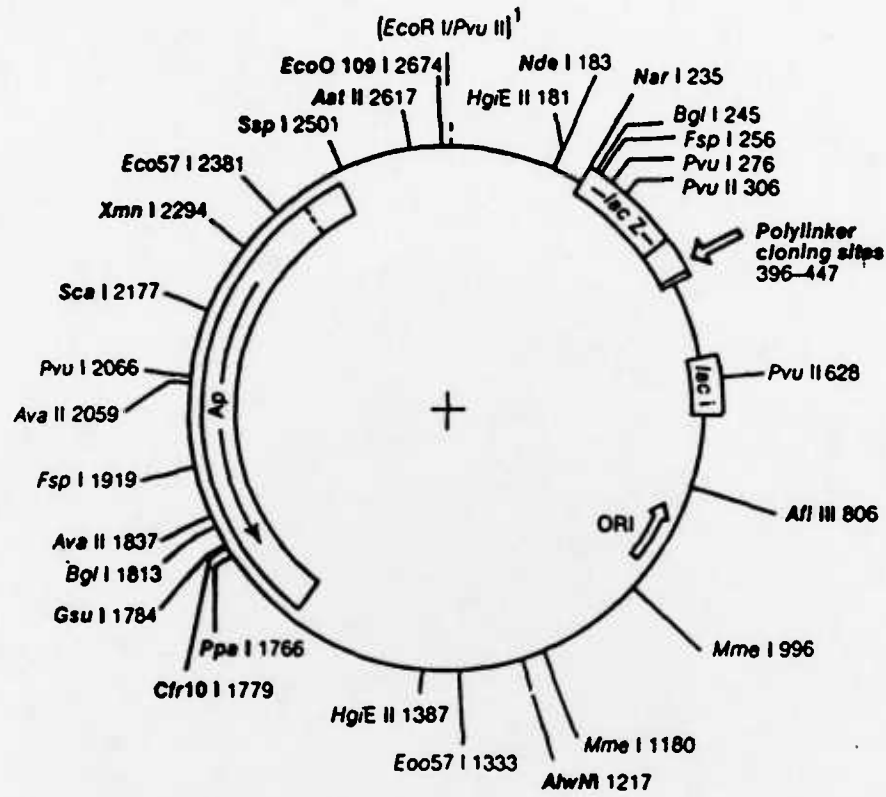
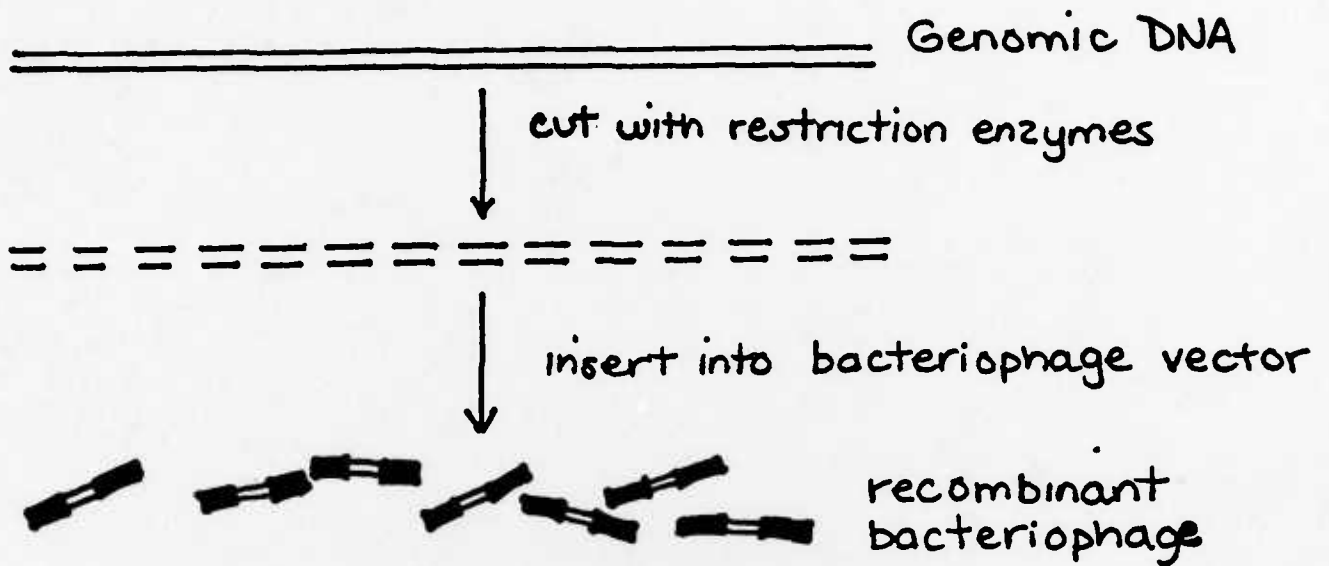


Fig. 4. pUC18 vector





~ 30,000 individual recombinant bacteriophage with 1000 bp inserts needed to represent falciparum genome of  $3 \times 10^7$  bp.

$\lambda$ gt 11 bacteriophage vector:

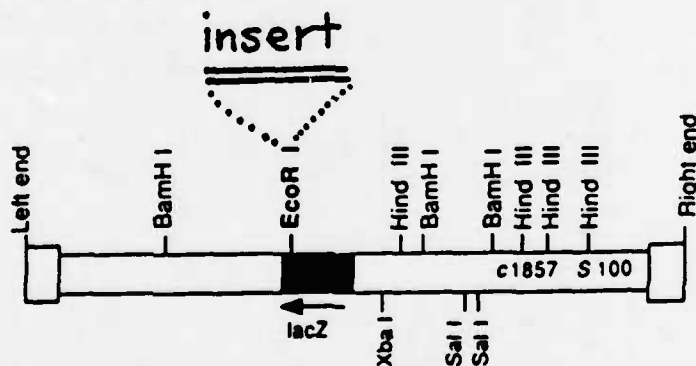


Fig. 5. Construction of a genomic DNA library for P. falciparum DNA polymerase alpha.

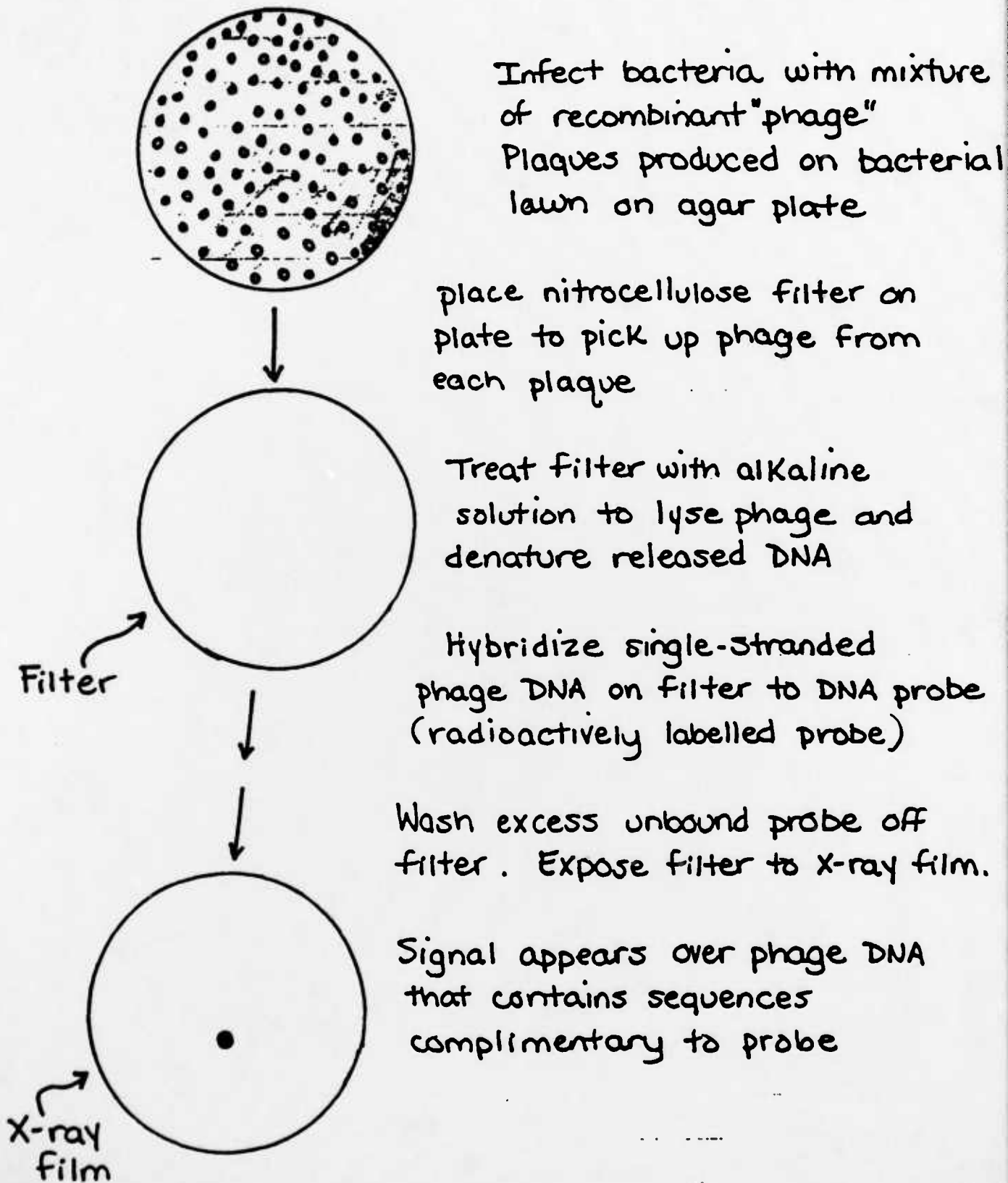
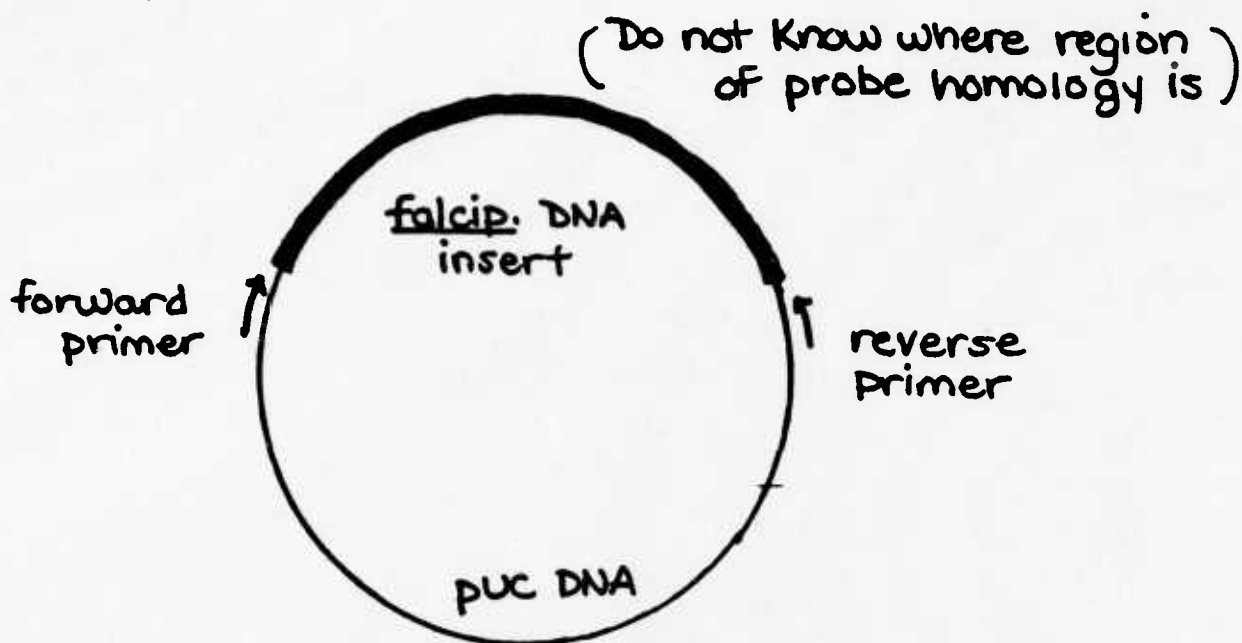


Fig. 6. Screening of the bacteriophage genomic library



### falcip. probe 1 inserts (4) -

- all same size -  $\sim 1.8$  Kb (1800 bp)
- inserts from 2 of the positives plaques (# 3 and 11) have been partially sequenced from the ends
- Clones 3 and 11 share same sequence, at least for 200-300 bp at the 5' and 3' ends
- No homology seen so far with probe 1, or with yeast or human polymerase DNA.

### falcip. probe 7 inserts (5) -

- range in size from 0.5 Kb - 3 Kb
- inserts from 3 positive plaques (# 16, 19, 21) have been partially sequenced from the ends.
- All 3 have different sequences, at least at ends
- No homology seen so far with probe 7, or with yeast or human polymerase DNA.

Fig. 7. Insertion of DNA from genomic library into pUC18 plasmids