CONSTRUCTION AND CHARACTERIZATION OF BACTERIAL ARTIFICIAL CHROMOSOME LIBRARY OF EGYPTIAN COTTON (*GOSSYPIUM BARBADENSE L.)*

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Abstract

BAC library for the Egyptian cotton *Gossypium barbadense* Giza 70, Giza 86, and Giza 75 varieties have been constructed and characterized. The isolation and purification of high molecular weight DNA from nuclei embedded in agarose microbeads was an essential part of this work. Several experimental parameters were investigated including optimization of megabase-size DNA restriction enzyme digest and CHEF gel conditions to achieve the highest resolution and separation of such DNA. Fragments ranging in size from 200Kb to 500Kb were size selected and recovered from agarose gel to be used in BAC library construction. The BAC vector, pBeloBAC II derived from the endogenous *E.coli* F-factor plasmid was used in library construction. Different insert: vector ligation ratios examined indicated 15:1 insert:vector to be the optimum ligation ratio. The maximum construct-host transformation efficiency calculated at 1.8KV electroporation voltage. Purification and isolation of BAC clones was followed by restriction enzyme digest using NotI to characterize insert sizes. Characterization of insert sizes and integrity using CHEF gel electrophoresis conditions at 6V/cm, 5 and 15 sec initial and final switch times respectively, and 12°C temperature for 12 hrs. BAC libraries for Giza 70, Giza 86, and Giza 75 varieties contained 45,237; 45,742; and 46,531 clones respectively with an average insert size of 100 Kb. Considering the haploid genome size for cotton to be 2,118 Mb, BAC libraries contains 2.12 haploid genome equivalent. Egyptian cotton BAC library constructed for *Gossypium barbadense* Giza 70, Giza 86, and Giza 75 varieties provide 88% probability of isolating a specific genomic region and are optimized for future gene identification, isolation, target gene mapping,and chromosome walking.

Introduction

Intense effort to construct a high resolution physical map of many plant crops is currently underway (Ahn & Tanksley, 1993; Coulson *et al.*, 1986; Kurata *et al.*, 1994; Zhang &Wing , 1997; Zwick *et al.*, 1998; Bush and Wise 1998). Eventually, these maps are composed of overlapping fragments of DNA and allow direct acquisition of DNA fragments that correspond to specific genes. Completion of the physical map requires the availability of comprehensive libraries of DNA clones in appropriate vectors. Furthermore, accuracy and efficiency of physical mapping increase progressively with the size of the cloned fragments in these libraries. While chromosome walking is straight forward in organisms with small genomes, it was difficult to apply it in most plant species which typically have large complex genomes in the absence of large-insert DNA libraries (Tanksley *et al.*,1995; Hong 1997; Zhu *et al.*, 1998).

Recently, some laboratories have explored the use of BAC cloning system for plants and animals (Woo S-S *et al.*, 1994; Kim U-J *et al.*, 1996; Danesh *et al.*, 1997; Yang *et al.*, 1997; Zhang and Wing 1997; Marek and Shoemaker 1997; Bent *et al.*, 1998; Miller *et al.*, 1998). It is emerging as the system of choice for plant genomic libraries with average insert sizes of 150 Kb. Cloning of exogenous DNA into bacterial artificial chromosomes (BACs) provides a new approach to the analysis of genomes of higher organisms (Shizuya *et al.*, 1992; Zhang and Wing 1997; Marek and Shoemaker 1997; Bent *et al.*, 1998). BAC libraries containing genomic DNA inserts are important tools for physical mapping, genomic sequencing and positional cloning. Since 1994, several human and plant BAC libraries have been constructed (e.g., s*orghum*: Woo S-S *et al.*, 1994; *arabidopsis*: Choi S *et al.*, 1995; rice: Zhang HB *et al.*, 1996; and human: Kim *et al* 1996, Danesh *et al.*, 1998). The average size of the plant libraries was 100 to 160 Kb. This system has advantages over both cosmid and YAC (yeast artificial chromosome) systems (Zhang, *et al.*, 1996; Bent *et al.*, 1998). Potential advantage of BAC system over YAC system include low level of chimerism, increased stability over high copy vectors (Kim, *et al.*, 1992), and ease of library generation and insert manipulation (O'Conner, *et al.*, 1989; Woo, *et al.*, 1994; Miller *et al.*, 1998). It has been demonstrated that BAC system has permitted cloning of DNA fragments up to 7-fold larger than cosmid system but it is much smaller than those of YACs (Shizuya *et al.*, 1992; Woo *et al.*, 1994; Zhang *et al.*, 1996; Zhu *et al.*, 1997).

BAC vector, pBeloBAC II, is derived from endogenous *E.coli* F-factor plasmid which contains genes for strict copy number control with unidirectional origin of DNA replication (Figure 1). Additionally, pBeloBAC II has three unique restriction enzyme sites (HindIII, BamHI & SphI) located within the LacZ gene which can be used as cloning sites for megabase-size DNA. The basis of high degree of stability of DNA fragments containing repetitive sequences is the single-copy number of vector (Kim *et al.*, 1992; Shizuya *et al.*, 1992; Iaonnou *et al.*, 1994). Results shown by Shizuya *et al.*,

Reprinted from the *Proceedings of the Beltwide Cotton Conference*
Volume 1:575-580 (2000)
National Cotton Council, Memphis TN

(1992) indicated that stable structure for cloned human DNA in the BAC vector represents a stable source of specific fragments. Very little or no rearrangement of the inserts or chimerism have been observed (Shizuya *et al.,* 1992; Woo *et al,*1994; Jiang *et al.,* 1995; Cai *et al.,* 1995; Zhu *et al.,* 1997). All current methods for isolating high molecular weight DNA resulted in a mechanical shearing of DNA with the largest fragments being about 50Kb (Couch 1990; Paterson *et al.,* 1993). Recently, techniques for the isolation of megabase-size DNA have been developed for a number of plant species (Ganal and Tanksley, 1989; Daelen *et al.,* 1989; Cheung and Gale, 1990; Sorbal *et al.,* 1990, Honeycutt *et al.,* 1992; Hatano *et al.,* 1992; Guidet and Langridge, 1992; Wing *et al.,* 1993; Zhang *et al.,* 1995, Frijters *et al.,* 1997). The DNA isolated by these techniques have been successfully applied to large-scale physical mapping for specific chromosome regions (Ganal *et al.,* 1989; Cheung *et al.,* 1991; Wing *et al.,* 1994; Zhang *et al.,* 1997; Zwick *et al.,* 1998; Bush and Wise 1998) and Map-based cloning of economical important genes in plant genomes (Arondel *et al.,* 1992; Martin *et al.,* 1992; Leyser *et al.,* 1993; Marek and Shoemaker 1997; Miller *et al.,* 1998).

In this work, we report the construction and characterization of BAC library for Egyptian cotton *Gossypium barbadense* Giza 70 (extra-long staple), Giza 86, and Giza 75 (long staple) varieties. Isolation of high molecular weight DNA from nuclei embedded in agarose microbeads, followed by optimization of megabase-size DNA restriction enzyme digest using HindIII enzyme are examined. Fragments ranging in size from 200 to 500Kb are targeted and recovered for BAC library construction using pulsed field gel electrophoresis. Different insert: vector ligation ratios are tested for optimization of ligation conditions. The maximum construct-host transformation efficiency is optimized using electroporation technique. Characterization of constructed BAC clones took place using pulsed field gel electrophoresis technique.

Materials and Methods

BAC Vector Preparation

pBeloBACII (7.4 Kb) in *E.coli* strain DH10B was provided by Dr. ***M.Simon,*** Cal Tech university (Figure 1). pBeloBACII vector was isolated as described by Woo et al. (1994) and Wang et al. (1995). Cells were harvested from an overnight culture in LB medium containing 12.5 mg/ml chloramphenicol (O.D.= 1.4). Plasmid DNA was isolated using the Plasmid Maxi Kit (Qiagen).

pBeloBAC II (20mg) was digested to completion with 3 units of HindIII (Biolabs) per microgram DNA at 37°C for 5 hrs. One mg aliquots of dephophorylated pBeloBAC II plasmid were stored at -80 °C until needed.

Plant Material and Megabase DNA Isolation

Cotton seeds of *Gossypium barbadense* Giza 70, Giza 86, and Giza 75 varieties were obtained from Coton Research Institute, Agricultural Research Center (ARC), Giza, Egypt. Seeds were grown in the greenhouse at Agricultural Genetic Engineering Research Institute. Young leaves were obtained from 4 to 6 weeks old plants, and placed immediately in liquid nitrogen. Cotton nuclie were isolated as described by Zhang *et al.* (1995). DNA homogenization buffer (1X) as desrcribed by Zhang *et al.* (1995) was substituted by cotton DNA extraction buffer as described by Paterson *et al.,* (1993). 0.5% Triton X-100 (w/w) was added immediately before use. Nuclie were pelleted at relatively low speed centrifugation (200g instead of 1800g).The nuclie were then embedded in agarose plugs (75ul of 1% agarose). Plugs were incubated in nuclie lysis buffer which consists of 0.5 M EDTA (pH 9.0), 1% (w/v) sodium lauryl sarcosine and 0.2 mg/ml protienase K (Boehringer Mannheim) at 50°C for 2 days. Nuclei lysis buffer was renewed after 24hrs. Nuclei suspended in TE buffer were stored at 4 °C. Prior to enzyme digestion, plugs were dialysed overnight against TE buffer plus 40mg/ml PMSF (phenyl methyl sulphonyl fluoride) at 4°C. The concentration of PMSF was increased to 80mg/ml followed by an additional incubation for 1h at room temperature to insure complete inactivation of proteinase K. Plugs were washed four times in TE for 30 min at room temperature. For partial digestions using Hind III, the plugs were equilibrated twice with Hind III restriction enzyme buffer for 1hr at room temperature. Plugs were melted at 65°C for 15 min. and held at 37°C for 5 min. For each plug (75ml), containing approximately 15mg DNA, 7 U HindIII was added and incubated for 45 minutes at 37°C. The reaction was stopped by the addition of 1/10 volume of 0.5 EDTA, pH 8.0. Partially digested cotton DNA was size fractionated on a low melting point agarose CHEF gel at 6 V/cm, with a 90s pulse, for 20h, at 11°C, in TAE buffer (50mM Tris-acetate, pH 7.7; 0.5mM EDTA pH 8.0) (Chu, *et al.,* 1986; Orbach, *et al.,* 1988). DNA ranging from 300 to 500 Kb was cut from the gel, and used for ligation or subjected to a second size fractionation. A second size selection was conducted for 16 h at 11°C running in TAE buffer, to generate a compression zone in excess of 1 Mb using a contour clamped homogeneous electric field (CHEF DR III apparatus, Biorad). The second size selection was carried out at 4.0 V/cm, with a 5s pulse in TAE buffer. The compressed DNA band was excised from the CHEF gel and stored at 4°C. A gel piece containing size selected DNA was melted at 67°C for 5 min. and digested with b-agarase (New England Biolabs).One unit of b-agarase per 100 ul of melted agarose gel was incubated at 40°C for 1hr.

Construction of BAC Library

After digestion, about 50 ng of size selected *Gosspium barbadense* genomic DNA was ligated to 750 ng dephosphorylated pBeloBAC II vector in a molar ratio of 1

(*G. barbadense* DNA) to 15 (vector DNA) in a total volume of 100ml. 400 unit T4 DNA ligase was added to the ligation mixture and incubated overnight at 16°C. Reaction was deactivated by incubation for 10 min at 65°C , then stored at 4°C until used. 15 ml of ligation product was transformed into 100 ml of electrocompetent ElectroMAX DH10B cells (Gibco BRL) using a Cell Porator system (Biorad) at the following settings: voltage:1.8; capacitance: 25mF; impedance Low ohms; charge rate: fast; Voltage booster resistance:200 ohms. Transformed cells were resuspended immediately in 1 ml of SOC medium (2%Bactotryptone, 0.5% Bactoyeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM Mg SO_4, 20 mM glucose, pH7.0), incubated at 37°C with shaking speed 225 rpm for 1 h and then plated on LB plates containing chloramphenicol (12.5mg/ml), X-GAL, and IPTG for 36-48 hrs. White recombinant BAC clones were picked with a toothpick to a second LB plus CM (12.5mg/ml), X-GAL, and IPTG plate to verify the color selection. White colonies from the second plate were then transferred to 96 well microtiter dish containing 200ml of LB freezing buffer (36 mM K_2HPO_4, 13.2 mM KH_2PO_4, 1.7 mM sodium citrate, 0.4 mM Mg SO_4, 6.8 mM $(NH_4)_2SO_4$, 4.4% (v/v) glycerol, LB). Microtiter dishes were stored at - 80°C for long term storage. The library was replicated in triplicate and stored in - 80°C.

Analysis of BAC Clones

Individual BAC clones were inoculated in 5 ml of LB broth containing 12.5 mg/ml chloramphenicol and grown at 37°C with shaking speed 250 rpm overnight. The circular BAC DNA was isolated by the alkaline lysis method used for the preparation of plasmid DNA (Silhavey *et al.*, 1984, Modified by Woo and Wing, 1993) . One quarter of the BAC DNA from the overnight culture was digested with NotI to release cotton DNA genomic insert from the cloning vector for at least 3 hrs. The digested BAC DNA was analyzed by PFGE with the CHEF III (BioRad, USA) using Lambda concatemer DNA ladder as the molecular weight marker. Conditions of the PFGE for the CHEF III were: 1% agarose gel, 0.5x TBE at 11°C, and 6.0V/cm for 12 h with linear ramping time from 5 to 15 s. The gel was stained with ethidium bromide and photographed. The insert size for each isolated BAC clone was determined.

Results and Discussion

Recently, techniques for isolation of megabase-size DNA have been developed for number of plant species (Zhao *et al.*, 1994; Zhang *et al.*, 1995; Frijters *et al.*, 1997). These techniques involve protoplasts or nuclei embedded in agarose plugs or microbeads. The agarose acts as a solid yet porous matrix which allows for the diffusion of various reagents for DNA purification and subsequent manipulations while preventing the DNA from being sheared. Most protocols for the isolation of megabase-size DNA from plants utilize the protoplast method (Raymond *et al.*, 1989; Wing and Michael 1990; Woo *et al.*, 1995). Although the protoplast method yields megabase-size DNA of high quality, yet the process is costly and labor intensive. Zhang *et al* (1995) have recently developed a nuclei method that works well for several divergent plant taxa. Nuclei are isolated and embedded in agarose plugs or microbeads. The quality of migrated DNA from agarose plugs is as good as DNA prepared from protoplasts, is often more concentrated, and was shown to contain lower amounts of chloroplast DNA. The primary advantage of this method is that it is economical and not as labor intensive as protoplast method. High molecular weight (HMW) DNA was prepared from Giza 70, Giza 75, and Giza 86 using the previous mentioned method with some modifications (Zhao *et al.*, 1994; Zhang *et al.*, 1995; Frijters *et al.*, 1997).

To test the quantity and quality of HMW DNA, megabase-sized DNA in agarose plugs was analyzed against DNA prepared by the conventional method (Figure 2A) by PFGE using *Saccharomyces cerevisiae* as a HMW molecular marker ranging from 1.9 Mb to 245 Kb in size. It was then evaluated in size, restriction enzyme digestibility, and yield. Genomic DNA was analyzed in a 0.6% chromosomal grade agarose gel (GibcoBRL) in 0.5x TBE for 72 hrs at 14 °C with 120° angle, 30 min pulse time and 1.5V/cm voltage gradient using a contour clamped homogenous electric field (CHEF) DRIII apparatus (Biorad) as described by Zhao *et al.*, (1994).The results indicated that the majority of cotton HMW DNA prepared from agarose embedded nuclie is retained in the well of the gel and in the compression zone which is located in a size range greater than 1Mb (Figure 2A). For comparison, the size of the cotton DNA prepared by a conventional method shows the majority of DNA between 50-200 Kb in size. There was no significant difference between megabase-size DNA embedded in plugs or in microbeads in yield or quality (Figure 2A). Microbeads were preferred over plugs in restriction enzyme digest because the use of microbeads increases the surface area surrounding the tissue sample by approximately 1000 fold thereby allowing for more efficient and rapid diffusion of chemicals and enzyme into and out of the agarose beads (Zhang and Wing 1997).

Digestion of HMW for Genome Analysis by PFGE

To determine the quality of HMW DNA in terms of its digestability, cotton DNA embedded in microbeads was partially digested using EcoRI and HindIII enzymes, and was subjected to PFGE. CHEF gel showed, several restricted bands on a smeared background for each restriction pattern (Figure2B). It was noted that all restriction fragments of HMW DNA generated with both enzymes were larger than 50 Kb. EcoRI generated fragments were in the size range between 200-450 Kb, while the size range of fragments resulting from partial digest using HindIII enzyme were in the size range between 200-600 Kb.

G. Barbadense BAC Library

pBeloBACII has three unique cloning sites within the b-galactosidase gene-BamHI, SphI, and Hind III. Previous experiments showed that HindIII restricts *Gossypium barbadense* DNA frequently with complete digest giving a size range below 50 Kb. To test the BAC system for library construction, *G.barbadense* DNA was partially digested with HindIII and the DNA fragments were size selected by pulsed field gel electrophoresis (Wing *et al.*, 1994; Zhao *et al.*, 1994; Zhang *et al.*, 1995). Figure 2B shows the optimal partial digestion conditions giving DNA in the size range of 200-500 Kb using HindIII. DNA between 300-500 Kb was isolated from this gel. In an attempt to increase the average insert size, a second size selection was made using a low melting point agarose gel, size selected at 4.0 V/cm, with a 5s pulse for 10 h, at 11°C. After electrophoresis, the compressed DNA band was excised from the CHEF gel, ligated to pBeloBACII vector and transformed into *E.coli*. Approximately 450 transformants per ml of ligation reaction was obtained which yielded 45,742 BAC clones per library.

Figure 4 shows the analysis of 12 BAC clones derived from insert DNA from two size selections. The DNA was digested with NotI to release the insert from the vector and resolved on a CHEF gel. The common band in all the lanes (7.4 Kb) is the BAC vector. The remaining bands are *G.barbadense* DNA fragments. Six of 12 inserts have a size in the range 97 to 145 Kb.One of the 12 inserts has an internal NotI site while 11/12 do not have an internal NotI site. A hundred and eight BAC clones were similarly analyzed. The average insert size per library ranged between 50-250 Kb.

The clones were grouped by insert size and the insert size of each clone was plotted versus the frequency of each group of clones presented in the library (Figure 3B). BAC libraries for Giza 70, Giza 86, and Giza 75 varieties contain 45,237; 45,742; and 46,531 clones respectively. The average insert size of the library is 100 Kb, which is equivalent to 2.12 haploid genome equivalent. The insert sizes of clones range from 50 to 250 Kb, and approximately 65% of inserts were in the size range between 81 to 145 Kb. Nearly 1.5% of the clones were in the size range between 146 to 250 Kb. Theoretically, the probability of obtaining a particular clone from this cotton BAC libraryis approximately 88%. We have generated a 2.12 haploid genome equivalent BAC librares for three Egyptian cotton varieties Giza 70, Giza 86, and Giza 75 using HindIII digest. There are a strong negative correlation between the numbers of colonies obtained and the average insert size. The cotton BAC libraries Giza 70, Giza 86, and Giza 75 contain 45,237; 45,742; and 46,531 clones with an average insert size 100 Kb comprising 4,523 Mb, 4,574Mb and 4,653 Mb DNA respectively from total cotton genome size (2118 Mb DNA).

Figure 3A shows a range of field strengths that was tested to determine the optimum electroporation conditions. Electroporation of *E.coli* strain DH10B electro-comptent cells (Gibco BRL) at 10 KV/cm resulted in a higher number of colonies per 15 ml ligation mixture which is less than the recommended settings of the Cell-porator system (12.5 KV/cm).

Critical Factors to be Considered in the Construction of BAC Libraries

The quantity and quality of input genomic DNA are two of the most critical factors. The BAC cloning protocols requires a series of compromises. While preparation of the genomic HMW DNA using the conventional method, it resulted in significant shearing of DNA. Optimization protocol isolation of the mega-base sized DNA embedded in agarose microbeads gave a satisfactory intact DNA profile. Sufficient DNA is required to compensate for the lower transformation efficiencies of large plasmids (Leonardo and Sedivy 1990; Sheng *et al.*, 1995; Wang *et al.*, 1995); nevertheless, if the pulsed field gels are overloaded, there will be excessive trapping of smaller DNA fragments on the PFG. These smaller molecules transform with much greater efficiency, and the average insert size drops dramatically. A significant amount of these smaller fragments can be excluded by a second size fractionation; this results in an increase in the average insert size of BACs (Frijters *et al.*, 1997).

Optimal concentrations of insert and vector are also the result of a compromise between conflicting requirements. Large genomic fragments have to be circularized with much smaller size plasmid. The importance of diluting the large fragments sufficiently to reduce the likelihood of intermolecular ligation has been demonstrated for jumping libraries (Collins and Weismann 1984; Poustka and Lehrach 1988). The larger the fragments to be cloned, the more they should be diluted. In addition, for the ligation of a small vector molecule to one end of the long DNA fragment followed by ring closure, the concentration of the vector should be roughly equal to the local concentration of the other end of the same molecule. However, diluting the large fragments results in a low concentration of circularized DNA fragments, and yields of transformants by electroporation are directly proportional to input DNA concentrations (Yuling *et al.*, 1995). Three ratios were tested 15:1, 1:10 and 1:15 (insert:vector) as (Choa *et al.*, in press). The greatest number of BACs per microliter ligation was obtained when approximately 50 ng large cotton fragments were ligated to 750 ng dephosphorylated pBeloIIBAC vector in a total volume 100ml. Based on considerations of stability, ease of isolation, yield, and size, the Egyptian cotton BAC library constructed in this study is suited for use in cotton genome sequencing, gene identification and isolation by chromosome walking. In addition, the library is appropriate for genome mapping and subsequent map-based cloning.

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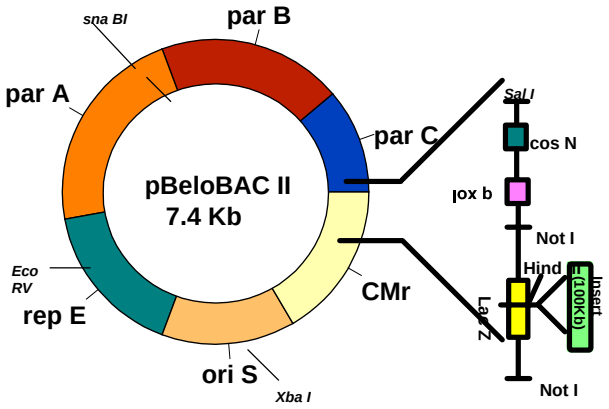

Figure 1. Diagram of pBeloBAC II. Size fractionated fragments of 250kbp of Hind III digested of HMW DNA of Egyptian Cotton varieties Giza 70, Giza 86 and Giza 75 were introduced to the HindIII site of pBeloBAC II vector. Insertional inactivation of the lacZ gene by the cloned DNA fragment results in white colonies after transformation.

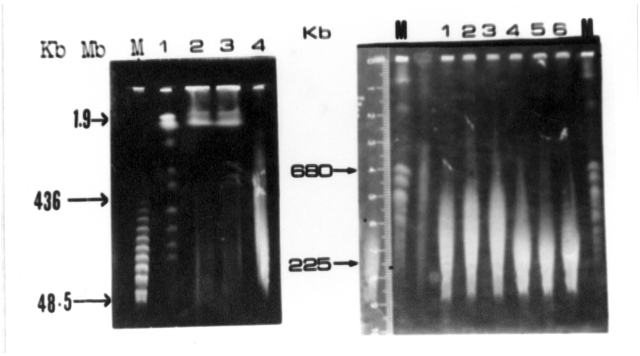

Figure 2

Panel A

Size Fractionation of Megabase DNA isolated from *Gossypium barbadense L.* using CHEF gel.
(M) lambda ladder PFG marker (Biolabs), lane (1)Yeast chromosome marker (Biolabs), lanes (2 and 3)HMW DNA of Giza 70 and Giza 86 repect., lane (4) conventionally isolated Giza 86 DNA.

Panel B

Restriction endonuclease digestion and size fractionation of megabase DNA from Egyptian cotton.
(M) Yeast chromosome marker (Biolabs), lanes (1,2 and 3) Partially digested DNA using HindIII Giza 70, Giza 86, and Giza 75 respectively, lanes (4,5 and 6) Partially digested DNA using EcoRI Giza 70, Giza 86, and Giza 75 .

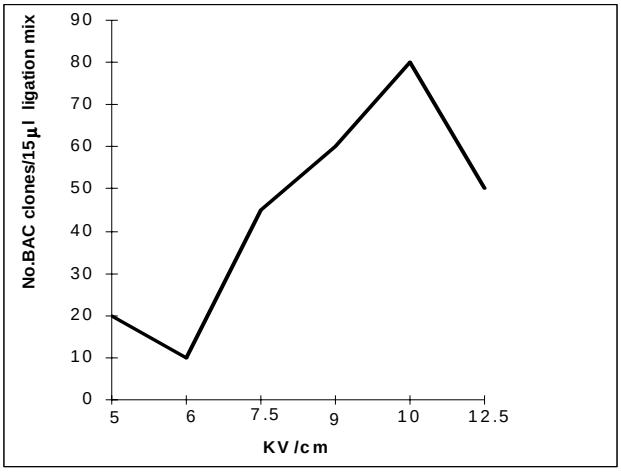

Figure 3

Panel A

Curve showing the relationship between field strength during electroporation and the number of transformants obtained.

15ul aliquot of ligation product was added to 100 ul of electro MAX DH10B cells (Gibco BRL) and placed between 0.2-cm gap electrodes. Different field strengths were tested with a constant pulse time of 6 mins.

The settings for the Cell-porator system (Biorad) were 1-2.5 KV; 25uF capacitor;and 200 ohms resistor.

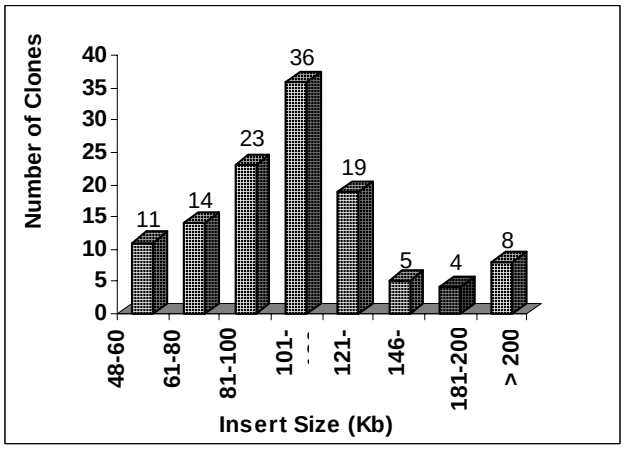

Figure 3

Panel B

A histogram showing the insert size distribution of BAC clones from Giza 70.

DNA was isolated from 120 random BAC clones and were size fractionated on CHEF gel.The clones were grouped by insert size and were plotted versus its frequency present in the library. Isolated clones have an insert size range from 50 Kb to 250 Kb. The average insert size of the Giza 70 BAC library is 100 Kbp.

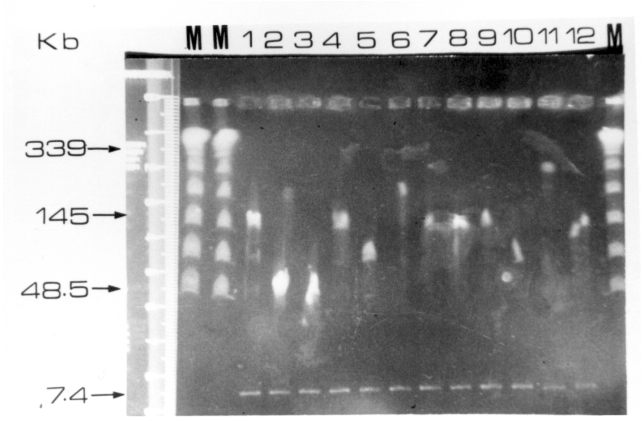

Figure 4. Analysis of Giza70 BAC clones. BAC clones were analyzed by pulsed-field gel electrophoresis (PFGE). The BAC DNA was isolated, digested with NotI to release the insert DNA from the cloning vector, and fractionated on a1% PFG. The 7.4 kbp band is the cloning vector pBeloBAC II. The insert size of each BAC clone is sum of sizes of all the bands of the lane (M) Lambda concatemer DNA ladder, lanes (1-12) BAC clones. The digested BAC clones was subjected to CHEF on a 1% agarose gel in 0.5 TBE buffer using a switch time of 5 to 15 sec. at 6 V/cm and 11°C with a 120° angle, for 12 hrs.