GENES AND GENE EXPRESSION: Localization, Damage and Control -
A Multi-Level and Interdisciplinary Study

(RENEWAL PROPOSAL ENTITLED: BIOPHYSICAL STUDIES OF HUMAN GENOME)

Progress Report

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Progress Report

Project I   3-Dimensional nuclear topography of genes and chromosomes in interphase nuclei

1.   Quantitation and Mapping of Integrated Human Papillomavirus DNA in a Human Metaphase Chromosome using a Fluorescence Microscope Imaging System

Integrated Human Papilloma Virus type 16 (HPV-16) DNA was directly visualized on metaphase chromosomes in the two human cervical carcinoma cell lines, SiHa and CaSki, by fluorescence *in situ* hybridization with a biotinylated DNA probe (7.9 kb). A single fluorescein signal, usually about four pixels in size, was observed on one or both chromatids of a large acrocentric chromosome in metaphase chromosome spreads from SiHa cells. A similar event was also observed in CaSki metaphase spreads; however, about eight to ten of the other chromosomes had bright multipixel fluorescent signals. Bright, multi-pixel fluorescent signals were also observed in all interphase nuclei of CaSki cells. In some cases, two small discrete fluorescence signals could be seen in some interphase nuclei of SiHa cells. Fluorescein hybridization signals were mapped on chromosome 13 which was identified by simultaneous hybridization with a biotinylated centromere probe specific for chromosomes 13 and 21. Ratios of the distance from 13pter to the HPV-16 signals to the entire chromosome length were approximately 0.63 ± 0.05 in both SiHa and CaSki cells, indicating the possibility of a common integration domain on chromosome #13. In SiHa cells, no additional signals were observed on other chromosomes. This observation, taken together with literature reports that SiHa cells contain one to two copies of the HPV-16 genome in this region of chromosome #13 suggests that each fluorescein signal on chromosome #13 represents one equivalent of the HPV-16 genome.

The integrated intensities of 25 individual fluorescent hybridization signals on chromosome #13 of SiHa cells were calculated and compared to the integrated intensities of all fluorescent signals observed in twelve separate CaSki spreads. This data show that the total integrated intensity in CaSki spreads was approximately two orders of magnitude greater than the integrated intensity of individual fluorescent signals on chromosome #13 of SiHa cells. The CaSki and SiHa cell lines are a good model system for validation of quantitative fluorescence imaging because the relative amount of HPV-16 in the two cell lines had been determined previously by Southern blot analyses and the integration site of a single copy of HPV-16 DNA had been mapped on SiHa cells to a defined site on chromosome #13. Our fluorescence data show that CaSki cells contain greater than 60 copies of the HPV-16 genome, which is in agreement with reports that this cell line contains 50 times more HPV-16 DNA than SiHa cells.

2.   Chromosome Topography of the Interphase Nucleus

a.   Simultaneous *in situ* hybridization and detection of three differentially labelled DNA probes

DNA probes specific for the following human alpha satellite DNAs were purchased from ONCOR, Inc., biotinylated chromosome #11, chromosome #17,

and chromosome #7. The chromosome #7 and #17 DNA probes were labelled by random primer extension with digoxigenin-11-dUTP and dinitrophenol-11-dUTP (DNP-11-dUTP), respectively. DNP-N-hydroxysuccinimide ester was prepared from DNP and N-hydroxysuccinimide and used to convert 5-(3-amino) allyl-dUTP to DNP-11-dUTP. *In situ* hybridization was carried out in 65% formamide-2x-SSC-10% dextran sulfate at 37° overnight using the instructions in a kit available from Oncor, Inc. After the washes of appropriate stringency, the hybridized probes were detected with red, green and blue fluorophores as follows: digoxigenin with a rhodamine-conjugated sheep anti-digoxigenin antibody; DNP with a goat anti-DNP antibody followed by a fluorescein conjugated rabbit anti-goat antibody; biotin was detected with avidin cascade blue followed by amplification with a biotinylated anti-avidin antibody and a second layer of avidin cascade blue. Figure #1 shows the detection of three different centromere probes in the same human lymphocyte; chromosome #7 centromere homologs are red, the #11 homologs are blue and the #17 homologs are green.

b. Optical sectioning and 3-D reconstruction of interphase human lymphocyte nuclei

The methodology described in the above paragraph was used for the simultaneous *in situ* hybridization of fixed human lymphocytes with three differentially labelled human alpha satellite DNA probes. Twenty-one of the stained nuclei were optically sectioned using our Zeiss Axiovert 35 microscope equipped with a Z-axis stepping motor. Using a 100x objective, stacks of 32 images at 0.23 micron increments were obtained for blue, green and red fluorescence emission with a DAPI, FITC and rhodamine filter combination, respectively. At this magnification, the pixel dimensions are 0.23 x 0.23 microns. Subarrays (64 x 64) of the monochrome images were flat-fielded and registered in both the "X,Y" plane and the "Z" direction. Registration of the images was facilitated by the fact that the red and green fluorescent centromeres, viz #7 and #17, respectively, were faintly visible after flat-fielding in the images taken with the DAPI filter set. Thus, due to the high sensitivity and dynamic range of the CCD camera and the excitation/emission characteristics of the blue filter combination, all six centromeres could be observed in images taken with the DAPI filter set. The registered images were overlaid to produce color composite images which were then assembled into a stack that provided 3-D information on the spatial distribution of the six hybridization signals (Fig. 2). It can readily be seen that centromere 11B is above the two chromosome 17 centromeres while 11A is below. The homologs of 7 are both in approximately the same plane and are below 11A and 17B. The distance of each centromere from the center of the labelled centromeres was determined as follows: The centroid (M), or the exact center of the six labelled centromeres, was calculated for each nucleus as the point of intersection of the median of a triangle formed by the midpoints of the three homolog pairs. The coordinates for the centromeres of each nucleus were transformed with respect to M being X = 0, Y = 0, Z = 0. The distance from M for all the centromeres was then calculated. The mean values, normalized to nucleus A, were 2.60 μ m, 3.08 μ m and 3.28 μ m for chromosomes 17, 11 and 7 respectively; these means are significantly different (P = 0.006, 1-way ANOVA). The mean distance of the centromeres from M is also related to the size of the chromosomes with 7>11>17.

c. Transformation of centromere coordinates

Our tricolor approach allows for the establishment of an internal reference frame to compare the spatial arrangement of chromosomes in different nuclei. The centromere coordinates from 21 different nuclei were mathematically transformed (to obtain a common frame of reference for each nucleus) as described below.

The coordinates for the center of mass (Cm) of the labeled centromeres was first determined as follows:

$Cm = 1/3 (Rm + Bm + Gm)$ for the X,Y and Z coordinates

where the midpoints:

$Bm\ X = 1/2 (B1X + B2X)$ and $BmY = 1/2 (B1Y + B2Y)$ etc.

where for example:

B1 and B2 are the two blue fluorescent chromosome 11 centromere homologs with X,Y and Z coordinates.

Therefore, using coordinates for:

R1, R2		Rm			
B1, B2	$\xrightarrow{\text{find}}$	Bm	$\xrightarrow{\text{find}}$	Cm	$\xrightarrow{\text{move}}$ Cm to X,Y,Z = 0
G1, G2		Gm			

To transform coordinates of centromeres:

Rotate O - Rm about the axis which is perpendicular to the Rm - O - X plane so that Rm goes onto the X-axis.

Rotate Bm about the O - X axis to bring it to the Z - O plane while keeping Rm on the X-axis.

After coordinate transformation:

Rm should be on X-axis
 Bm and Gm should be on $Z = 0$ plane
 Cm should have coordinates $X, Y, Z = 0$

The transformed coordinates (X,Y,Z) for all eight centromeres from 21 different nuclei were plotted to determine the extent of clustering of each homolog. When the transformed data were plotted, a non-random spatial distribution of chromosomes 7, 11 and 17 was observed. The data for chromosome 7 is shown in Figure 3. Centromere pairs were seen to occupy common domains within the interphase nucleus and each pair of homologs appeared to be restricted to a specific region of the nucleus. The volumes, with respect to the average nuclear volume, that 75% of the 21 centromere homologs can encompass is 4.3 to 7.2% for chromosome 17; the values for chromosome 11 and chromosome 7 are 11.2 to 11.4% and 17.6 to 22.5%, respectively. Intranuclear landmarks such as the nucleolus or the membrane are not sufficient to discern the chromosome arrangement described above.

3. Chromosome Structure During Cell Cycle

Using a whole chromosome painting probe available from GIBCO, we have painted chromosome 4 in interphase, prophase and metaphase. The DNA in normal metaphase chromosomes is highly compacted while the chromosome arms are much more extended in prophase. In interphase the chromosomes occupy discrete domains; upon optical sectioning and 3-D reconstruction, the extended chromosome arms appear to fold back on themselves, thus forming a discrete domain.

Fig. 1

Computer-generated composite image showing three probes in one interphase nucleus. Biotinylated chromosome 11 centromeres are stained blue, digoxigenin-labeled chromosome 7 centromeres are stained red and DNP-labeled chromosome 17 centromeres are stained green.

Fig. 2

Three-dimensional reconstruction of 6 chromosomes centromeres in one interphase nucleus using data from tricolor stack of 32 images.

Fig. 3

Three-dimensional view of chromosome 17 data from 21 different interphase nuclei showing the clustering of the two homologs. The coordinates were mathematically-transformed before being plotted.

Progress Report

Project II   Sequence specific identification and perturbation
of the genomic DNA in living cells by nonionic
oligonucleotide analogs (Matagen)

A.   Progress in the general studies on nonionic oligonucleotide analogs

A most recent review on this subject will be soon published in the Annals of New York Academy of Sciences (Appendix No. 3). In general, three comments should be made:

(1) This field on antisense/anticode has now received a great deal of attention and enthusiastic participation. The meeting in January, 1991 entitled "Antisense Strategies" was very well attended (several hundred conferees). Dr. Ts'o and Dr. Miller are the organizers of the "International Conference on Nucleic Acid Medical Applications" to be held in Cancun, Mexico, January 1993. Currently, the response has been also very enthusiastic.

(2) One of the spectacular findings is the work of Professor Esther Chang's group in collaboration with us that Matagen of 11 units in length can recognize a single base change in the mRNA (Appendix No. 4). Using the normal human Ha-ras p21 system as a model, a single change of the base (i.e. from A to T) can be distinguished by the Matagen in the living cells. As quoted from the abstract of this paper, "Antisense oligonucleoside methylphosphonates and their psoralen derivatives directed at either normal human Ha-ras p21 or ras p21 that is mutated at a single base in codon 61 have been examined for their efficacy and specificity as inhibitors of p21 expression. Mixed cultures of cells expressing both forms of p21 were treated with the antisense oligomer complementary to the normal p21 or with the antisense oligomer complementary to the point-mutated p21. Each of the antisense oligomers specifically inhibited expression of only the form of ras p21 to which it was completely complementary and left the other form of p21 virtually unaffected".

(3) The work of Professor Laure Aurelian's group in the University of Maryland in cooperation with our laboratories, have clearly indicated the sequence-specific inhibition of the splicing of the mRNA of HSV-1 (1). The results provide concrete evidence that Matagen can enter the nucleus of the mammalian cells and inhibit the splicing process taking place inside the nucleus (see Appendix No. 3). This work and others to be reported here provide the foundation for the experimental design in perturbation of the genomic DNA in the nucleus by Matagen via triplex formation.

B.   Triplex formation with the third strand of oligomer consisting of
methylphosphonate backbone

Triplex formation among 3 strands of deoxyoligonucleotides (regular phosphodiester backbone) have been investigated extensively in our laboratories by UV, CD, fluorescence and proton NMR spectroscopy (2). This work provides a foundation for the comparative CD and fluorescence studies of the oligodeoxyribonucleotide and oligodeoxyribonucleoside methylphosphonate pyrimidine strands in duplex and triplex formation (Appendix No. 5). A copy of the reprint is included in the Appendix. The experimental work is

corroborate by the molecular dynamics simulation. After extensive computation on oligonucleoside methylphosphonates (MP) and naturally occurring oligodeoxynucleotides (ODN) as the third strand to double-stranded (ds) DNA targets predict that the third strand with MP backbone is more favorable to triple helix formation than the native phosphodiester ODN third strand (3). In contrast to experimental data, the calculated DNA conformations in both fully solvated triple helical system are found to be in hybrid A and B conformations. The calculations predict that a third strand with MP backbone is readily accommodated in the major groove of the ds DNA target, and adopts a different conformation from a helical triplet formed with native ODN as the third strand through the Hoogsteen base pairing scheme (3).

C. Triplex formation of oligonucleotides containing 2'-O-methylpseudoisocytidine in substitution for 2'-deoxycytidine

In the triplex formation with pyrimidine-purine-pyrimidine motif, the T/A-T can be formed at neutral pH, but the C'/G-C has to be formed at acidic pH because of the requirement of protonation of cytosine moiety. Our laboratory has synthesized a new nucleotide analog, 2-amino-5-(2-O-methyl- β -D-ribofuranosyl)-4(1H)-pyrimidine (or 2'-O-methylpseudoisocytidine) as a replacement of 2'-deoxycytidine in the oligonucleotides (Appendix No. 6). As shown in the following Fig. 1, the analog contains an NH at the N3 position. Therefore the requirement of protonation of N1 of cytosine has been eliminated. In these papers, the synthesis of the oligonucleotide containing this analog have been reported. In addition, the formation of triplex by this pseudoisocytidine containing oligonucleotide with short DNA duplex for two separate sequences were reported. These two papers are included in the Appendix. Most recently, oligonucleotide methylphosphonate containing the 2'-O-methyl pseudoisocytidine have just been synthesized. In a preliminary study, this oligomer behaves as predicted in the formation of a stable triplex. Thus at present, the third strand of methylphosphonate oligomer containing T (or 2'-O-methyl U) and pseudoisocytosine is expected to form a stable triplex with homopurine-homopyrimidine sequence with a motif of T/A-T or pseudoiso C/G-C.

D. Triplex formation of nonionic oligonucleoside methylphosphonate with short duplex of DNA in a purine/purine-pyrimidine motif.

As mentioned above, the triplex formation of pyrimidine/purine-pyrimidine motif has been extensively investigated. On the other hand, triplex formation consisting of three strands of negatively charged poly- or oligo-nucleotides in a purine/purine-pyrimidine motif is much less known and has been found to be unstable or unobservable. Recently, in our laboratory with the third strand consisting of nonionic methylphosphonate backbone, triplex with purine-purine-pyrimidine was found. Using the homopurine oligomer, $d(AG)_8$, its nonionic methylphosphonate analog, $d(\underline{AG})_8$, and the complementary homopyrimidine oligomer, $d(CT)_8$, as a model system, the stoichiometry, conformation and stability of complexes formed at pH 8 are studied by UV and CD spectroscopies and native PAGE. When there is only one oligomer species in solution, $d(\underline{AG})_8$ behaves as a single-stranded molecule. In contrast, the $d(AG)_8$ oligomer readily forms a self-complex, particularly in the presence of magnesium ion. Duplexes can form with the $d(CT)_8$ oligomer using either homopurine oligomer which differ in terms of their conformation and in the dependence of their thermal stability on sodium and magnesium ions.

All studies show that a stable triplex forms with 2:1 d(AG)₈:d(CT)₈ stoichiometry which does not require high concentrations of sodium or magnesium ions. Triplex formation between two d(AG)₈ strands and the d(CT)₈ strand was not detected at any condition. Native PAGE studies suggest that a 1:1:1 d(AG)₈:d(AG)₈:d(CT)₈ complex may be formed. In regard to triplex formation, the advantage of the methylphosphonate backbone on the purine strand is clearly demonstrated (Appendix No. 7). In addition, using the 16-mer model system, dA₁₆-dT₁₆, and the corresponding nonionic methylphosphoate backbone analogs, dA₁₆-dT₁₆, we investigated the complexes which formed at pH 7 in 0.1M NaCl by CD spectroscopy and UV mixing and melting curves (Appendix No. 8). Hybrid methylphosphonate-phosphodiester duplexes (A•T and A•T) and triplexes (A•A•T, T/A•T, and T/A•T) were observed and characterized. The parent phosphodiester oligomers formed an A•T duplex (T_m = 36°C), but no complex of higher strand stoichiometry was observed at the same conditions. The dA•dT hybrid duplex has a T_m of 47°C. The A/A•T and T/A•T triplexes also have T_ms of 47°C. Complex formation by the nonionic oligomers at moderate ionic strength may be enhanced by reduction of charge repulsion and possible increased flexibility of the methylphosphonate backbone, especially when more than two strands are involved. The study of the A-A•T triplex is particularly important in view of the dominant role played by G-G base pairs in pur•pur•pyr triplex formation by strands having phosphodiester backbone.

These two studies strongly suggest the presence of A/A•T and G/G•C motif when the third strand is A or G oligomers consisting of nonionic backbone. The presence of these purine•purine-pyrimidine motifs is under investigation by NMR (Appendix No. 7).

E. Triplex formation between psoralen-derivatized oligothymidine methylphosphonates and DNA (Appendix No. 9)

The interactions between 4'-(aminoalkyl)-4,5',8-trimethylpsoralen [(aa)AMT] derivatized oligothymidine methylphosphonates and a double-stranded DNA 45-mer target were studied in vitro. The 45-mer contained a tract of 15 A-T base pairs within the sequence (nucleotides 8-22). This tract was flanked on either side by T-A base pairs which provide crosslinking sites for the psoralen group. Oligomers, d-(aa)AMTpTp(T)₆ and d-(aa)AMTpTp(T)₁₃, where the underline indicates the presence of methylphosphonate linkages, crosslink with the DNA target when a mixture of the target and excess oligomer are irradiated with 365 nm ultraviolet light. Crosslinking occurs with the T₁₃ containing strand but not with the A₁₅ containing strand and occurs to a greater extent with oligomers having a butyl (ab) or hexyl (ah) linker as demonstrated by the mobility shift of the photoadducts on polyacrylamide gels run under denaturing conditions. The extent of crosslinking by d-(ab)AMTpTp(T)₆ to the 45-mer, whose binding site can accommodate two molecules of the oligomer, was 45% at 4°C, whereas 23% crosslinking was observed with d-(ab)AMTpTp(T)₁₃. These results are consistent with the formation of covalent adducts between the psoralen-conjugated oligothymidine methylphosphonates and the A-T tracts in the DNA as a result of triplex formation.

F. Uptake of oligonucleoside methylphosphonates derivatized with rhodamine

A 3'-rhodamine-derivatized oligonucleoside methylphosphonate whose sequence is complementary to part of the coding region of vesicular stomatitis virus (VSV) M-protein mRNA was prepared and its uptake by mouse L-cells and

VSV-infected mouse L-cells was investigated using fluorescence microscopy. The oligomer appears to be taken up by the cells in a manner similar to that of previously reported for [H^3]-labeled oligomers. Most of the fluorescence appears to accumulate in vesicles similar to endosomes in the cytoplasm of the cell. A more diffuse fluorescence is seen throughout the cytoplasm and nucleus. It does not appear that the oligomer "sticks" to the cellular membrane and the oligomer is stable in the cell culture medium. The oligomer does not appear to wash out of the cells when the oligomer-treated cells are incubated with oligomer-free medium even after three days of incubation.

A rhodamine-derivatized oligomer described above was further derivatized at its 5'-end with 4'-(aminoethyl)-4,5',8-trimethylpsoralen [(ae)AMT]. This oligomer was shown to crosslink *in vitro* to single-stranded 19-mer RNA and DNA targets with an efficiency equal to or slightly greater than that of the singly derivatized (ae)AMT-derivatized oligomer. We are currently studying the uptake of this psoralen-derivatized-rhodamine-derivatized oligomer in mouse L-cells and in mouse L-cells infected with VSV.

G. Constant activity gel electrophoresis (CAGE)

Gel electrophoresis at native conditions is a method commonly used to detect complex formation in oligonucleotide systems. The method relies upon the differential mobility of a labeled species as a free strand or bound in a specific complex. For interactions between oligonucleotides having charged phosphodiester backbone, electrophoretic mobility usually decreases in the order of single- > double- > triple-strand. For hybrid complexes involving phosphodiester and methylphosphonate oligomers, mobilities of duplexes and triplexes will be less than those of the respective parent complexes as a result of having one or more of the interacting strands with nonionic backbone. A free methylphosphonate oligomer is expected to have no significant mobility during electrophoresis. The widely different mobilities of charged phosphodiester target strands and nonionic methylphosphonate probe strands is a major problem when trying to detect complex formation by such methods. If a hybrid complex should dissociate at any time during electrophoresis, the single-stranded components will be rapidly separated by the electric field, resulting in an infinite dilution of the interacting species within the gel. It should be noted that this phenomenon is usually not a major problem for interactions between phosphodiester oligomers, when the relative mobilities of two charged single strands will be approximately equal. Thus the two separated single strands will remain in the same region of the gel even though they do not reside together as a complex. Under such conditions, the complex will be able to reform as the interacting oligomers have maintained an effective concentration in the gel.

In order to overcome the difficulty of detecting hybrid phosphodiester-methylphosphonate complexes by native gel electrophoresis, a system can be devised in which the concentration of nonionic species remains constant. This is achieved by forming the gel matrix in the presence of a desired concentration of methylphosphonate oligomer and then electrophoresing a pre-established equilibrium solution containing the labeled, charged target oligomer and the nonionic probe. In this case, whenever a dissociation event occurs, the original uncharged ligand will remain behind in the gel. However, the concentration, or activity, of this species is constant throughout the matrix; therefore, the probability of an association event according to the

Constant Activity Gel Electrophoresis - CAGE

Conditions: 0.1M NaCl, pH 8 (TPE buffer)

15 °C, 10 V/cm, 12 hrs

original equilibrium is maintained. We are in the process of developing this constant activity gel electrophoresis (or CAGE) method. Using a system of specially designed combs, several zones containing different concentrations of methylphosphonate oligomer may be cast within the same polyacrylamide gel. Shown in Figure 1 is a CAGE gel in which labeled $d(CT)_8$ oligomer has been electrophoresed into zones containing different stoichiometries of nonionic $d(AG)_8$. The band in lane 1 represents the mobility of the single-stranded phosphodiester oligomer. At increasing $d(AG)_8:d(CT)_8$ ratios (lanes 2-5) the presence of hybrid $d(AG)_8 \cdot d(CT)_8$ duplex and $d(AG)_8 \cdot d(AG)_8 \cdot d(CT)_8$ are readily detected. The CAGE method may also be used to determine binding constants at isothermal conditions. When labeled $d(AG)_8$ is electrophoresed at 35°C in 1:1 stoichiometry with $d(CT)_8$ over a range of concentrations in a constant activity gel (Fig. 2), an equilibrium binding isotherm is obtained. The association constant derived from this data is $2 \times 10^7 M^{-1}$ for formation of the hybrid $d(AG)_8 \cdot d(CT)_8$ duplex. This value compares very favorably with a value estimated at 35°C ($K_{\text{assoc}} = 1 \times 10^7 M^{-1}$) from standard optical melting curves.

H. Covalent triplex formation with Matagen in living mammalian cells

In addition to the biochemical evidence, the above section F provides microscopic - cytological evidence of the uptake of Matagen into the cell and nuclei. Also in section E, chemical evidence has been presented to indicate the photo-induced crosslinking of T-Matagen derivatized with psoralen with the dA-dT short helices.

Thus, we have done preliminary data to indicate the feasibility of photo crosslinking of T-Matagen derivatized with psoralen into genomic DNA, presumably onto the $(dA-dT)_n$ region. We first determine the totally available site by protein removal and DNA denaturation. Then we determine the portion of $(dA-dT)_n$ region which is not covered or protected by protein. Finally, we have characterized the product with $(^{32}P)(pT)_6$ -psoralen-DNA by a series of washing, RNAase, proteinase K, and DNAase treatment and with photo-induced reversal of linking up irradiation at 254 μM . The experimental procedure and results are outlined below:

Binding of psoralen- $^{32}P(pT)_6$ to fixed and live mammalian cells.

Hamster embryo cells were fixed with methanol-acetic acid (3:1), extracted with cold 0.05 N HCl to remove histones, treated with RNase and proteinase K. DNA was denatured *in situ* with 0.15 NaOH-70% EtOH. After neutralization and washing, the cells were incubated with ^{32}P -labeled-oligothymidylate methylphosphonate (7 mer) at 4° for 2 hr; then 1/2 of the preparations were irradiated with 365 nm light for 15 min. After washing with CH_3CN-H_2O (1:1), the cells were stained with DAPI, coverslips mounted and the number of cells per chamber was determined with a fluorescent microscope. The coverslip was removed and the cells dissolved in 0.5 ml of 0.1 N NaOH. After neutralization the number of oligomers bound per cell was determined by scintillation counter. In the above situation, where the DNA was denatured and RNA and protein removed, there were 2.2×10^6 thymidylate methylphosphonates bound per cell. This would represent the total number of binding sites in genomic DNA. In a second experiment, the cells were fixed and treated with RNase, but protein was not removed, nor was the DNA denatured. Under these conditions, 4 to 5×10^5 molecules of $^{32}P(pT)_6$ were bound after irradiation. This should represent the number of triplexes formed

origin →

NC →

RpH →

8.5 8.25 8 7.75 7.5 7.25 7 6.75 6.5

*d(AG):d(CT) 8:1, CAGE at 35°C

0.1M NaCl, 2mM PO_4^{3-} , 1mM EDTA, pH 7
15 V/cm, 200 mA, 1.7 hr.

in chromatin of fixed cells. In a third experiment, live cells were incubated for 2 hr with the ^{32}P -labeled oligomer at 37°C . The cells were placed on ice for 10 min, medium removed and replaced with Hank's balanced salt solution. They were then irradiated for 15 min with 365 nm light. The cells were fixed and washed with $\text{CH}_3\text{CN}-\text{H}_2\text{O}$ (1:1). Under these conditions, $\sim 1 \times 10^6$ molecules of oligomer were bound per cell. This should represent the number of triplexes formed in chromatin plus the number of binding sites in cellular RNA. This data is very encouraging and we are now ready to investigate this problem by a sequence-specific attachment onto a defined DNA target in the living cells.

Triplex Formation with Psoralen - Matagen in Human Fibroblast

*(Background: Oligomers addition without 365μm Irradiation)

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Progress Report

Project III   Resolution and isolation of specific DNA restriction fragments

As described in the general introduction, much of the efforts in the past 5 to 6 years have been devoted to the development of techniques/procedures, so that one or several specific DNA restriction fragments can be resolved, identified and isolated from a large population of restriction fragments.

1.   2-Dimensional Agarose Gel Electrophoresis.

(1) About 6 years ago, our laboratory began to utilize the specific advantage in gel separation technology by using electrophoresis to conduct physical chemical reactions or enzymatic reactions of each DNA restriction fragment confined in a small space of the gel matrix following size separation. In essence, we can conduct many experiments at once in a gel for all these individually sized (or charge) separated DNA fragments. The first results of ours, published in 1989, was "A General Method for Detecting Rearrangements in a Bacterial Genome" by utilizing the denaturation and renaturation of two samples of DNA (1:1000 mass ratio of *E. coli*- λ DNA versus *E. coli* DNA) *in situ*, within the gel, after first dimensional separation (ref. 1). In this process, the labeled inserted DNA (λ) fragments from the test cells will renature much slower, as compared with the commonly shared DNA fragments, since in this location of the gel these inserted or rearranged fragments (λ DNA) do not have a counterpart in the driver DNA provided by the normal *E. coli* cell which is in excess by about 1,000 fold. After renaturation, the DNA in the gel is electrophoresed through a second dimension which is perpendicular to the first dimensional separation's direction. The denatured, inserted DNA fragments are revealed after autoradiography, since single-stranded, denatured DNA fragments have higher electrophoretic mobility than double-stranded fragments (after renaturation) of the same sizes. The reason for describing this earlier paper is that the *in situ* denaturation/renaturation process is one of the crucial steps now being adopted in the development of 3-D gel electrophoresis for affinity separation.

(2) In the next stage of development, again utilizing the principle of conducting in-gel reactions with individually size-separated DNA fragments, we add a second restriction enzyme for digestion of these DNA fragments individually, within the gel. This approach resulted in a publication entitled "Enhanced Resolution of DNA Restriction Fragments: A Procedure by Two-Dimensional Electrophoresis and Double-Labeling" (ref. 2).

A probe-free method was developed to detect DNA rearrangements in bacteria based on the electrophoretic separation of twice-digested restriction fragments of total genomic DNA into a two-dimensional (2-D) pattern. The first restriction enzyme digestion was done in solution, followed by electrophoresis of the restriction fragments in one dimension. A second restriction enzyme digestion was carried out *in situ* in the gel, followed by electrophoresis in a second dimension perpendicular to the first electrophoretic separation. The 2-D pattern provides for the resolution of 300 to 400 spots, which are defined

and indexed by an "x,y" coordinate system relative to size marker standards. This approach has greatly increased the fragment resolving power over conventional one-dimensional (1-D) electrophoresis. To study DNA rearrangement, a 2-D pattern from a test strain was compared with the 2-D pattern from a reference strain. After the first digestion, genomic DNA fragments from the test strain were labeled with ^{35}S , while those from the reference strain were labeled with ^{32}P . This was done to utilize the difference in the energy emissions of ^{35}S and ^{32}P isotopes during autoradiography when two x-ray films were exposed simultaneously on top of the gel following the 2-D electrophoretic separations. The irradiation from the decay of the ^{35}S atoms exposed only the lower film, whereas the irradiation from the decay of ^{32}P atoms exposed both the lower and the upper films. Different DNA fragments existing in the test DNA, compared with the reference DNA, can be identified unambiguously by the two differential 2-D patterns produced on two films upon exposure by the ^{35}S and ^{32}P fragments in a common gel. An appropriate photographic procedure further simplified the process, allowing only the difference in DNA fragments between these two patterns to be shown in the map. We have utilized the difference map obtained from *Escherichia coli* strains HB101 and HB101(λ) genomic DNA to show the incorporation of one copy of phage lambda DNA without the use of a lambda DNA probe. This is the basic process utilized in the first phase of the 3-D gel electrophoresis procedure.

(3) With the realization that this method is insufficient to separate the millions of restriction fragments obtained upon the digestion of the mammalian genome after the digestion by two restriction enzymes, we developed a probe which has several hundred copies interspersed throughout the genome. This probe can be used for monitoring polymorphisms and rearrangements that may exist involving a specific mammalian gene. Genome rearrangement(s) involving mid-repetitive sequences, such as the intracisternal A particle (IAP) gene, and their neighboring sequences in rodent cells can be monitored by using these mid-repetitive sequences as probes (ref. 3). This can be done by performing electrophoretic separation of twice digested restriction fragments of genomic DNA, via 2-D electrophoresis, into a 2-D plane. The first digestion was done in solution followed by electrophoresis of the restriction fragments in the first dimension. A second restriction enzyme digestion was carried out *in situ* in the gel followed by electrophoresis in a second dimension perpendicular to the first electrophoretic step. After Southern transferral to a membrane, the filter-bound DNA is hybridized with a probe that is a fragment located near the 5'-end of the IAP gene, but which does not overlap with the 5' long terminal repeat (LTR) sequence. The exposed X-ray film revealed about 370 distinct spots in the 2-D maps. In comparing the 2-D maps, genome rearrangement involving IAP was detected.

(4) After this successful demonstration of the IAP-probe on the Syrian hamster genome, we launched an ambitious attempt to study the polymorphism of the human genome utilizing the repetitive, transposable, LINE-1 element as probe. For this application, we utilize a large scale agarose gel (Appendix 10). We have developed a Southern blot method with high resolution power to resolve a class of interspersed, repeated sequence in human genome. This method applies two-enzyme digestions including *in situ* digestion and two-dimensional electrophoresis on a large sized agarose gel (40 cm X 50 cm). Using the very 5'-end of the long interspersed repeated DNA sequence of LINE-1 as a probe, two individual human genomic DNA samples from placenta were

analyzed. About 900 LINE-1 spots were resolved from each human genomic DNA on the two-dimensional plane in the size range between 1 to 23 kb and minimally 15 polymorphic spots were detected through visual examination between two individual genomic DNA samples. After the two-dimensional electrophoresis, considerable amounts of LINE-1 spots still stayed on the diagonal line owing to the absence of restriction enzyme sites in these restriction fragments. In order to analyze these remaining spots, we have developed an optional procedure that separates the diagonal line fragments, from the rest of the gel, and treating this gel strip with a third restriction enzyme. About 380 additional LINE-1 containing spots were resolved from the DNA fragments originally on the diagonal line. This optional procedure can be repeated to resolve more spots for further analysis in the 2-D plane until the LINE-1 spots on a diagonal line become resolvable. These methods have been applied to monitor rearrangements adjacent to the interspersed sequences in the human genome.

(5) Another exciting application of the 2-D and 2-enzyme gel electrophoresis process is for constructing linear fragment maps of 50 kb to 100 kb sized DNA molecules (Appendix 11). A simple and effective procedure to construct linear restriction fragment maps was developed. Using a two-enzyme digestion two-dimensional (2-D) electrophoresis procedure, in both forward and backward order, each double-digested fragment contains the information for defining the overlap of the parental, singly digested fragment obtained from the first enzyme as well as the parental, singly digested fragment obtained from the second enzyme.

All the restriction fragments in a 50 kb to 100 kb sized DNA molecule can be individually resolved and displayed on a 2-D plane. This 2-D gel pattern, with appropriate markers, provides a fixed set of x,y coordinates for each fragment obtained from the single and double digestion as well as the relationship between the two steps.

This is obtained first by a forward order of enzyme treatment (i.e. enzyme-1 first, then enzyme-2), then with a backward order of enzyme treatment (i.e. enzyme-2 first, then enzyme-1). A matrix is constructed from the 2-D pattern obtained from the forward order enzyme treatment as well as from the 2-D pattern obtained from the backward order enzyme treatment. The vertical column shows all the singly digested individual fragments and their sizes obtained from each restriction enzyme treatment, and the dividing horizontal row shows all the doubly digested DNA fragments and their sizes after treatment with two enzymes. The order of arrangement is always from the smallest to the largest fragments.

Using this matrix, two linear DNA restriction maps for these two enzymes can be simultaneously constructed in a self-reconfirming manner. As examples for this procedure, we describe the construction of two linear restriction fragment maps, a combination of *Eco* RI and *Bam* HI digestion as well as a combination of *Eco* RI and *Hind* III digestion of lambda-phage DNA.

After the test case with lambda DNA, we went on to construct the restriction map for a 40 kb cosmid containing human DNA from chromosome 21 (Appendix 12). All the DNA restriction fragments up to 15 kb in size are individually resolved and displayed on a 2-D plane through a two-enzyme

digestion, two-dimensional (2-D) electrophoresis approach. In addition, a sensitive method to label DNA fragments *in situ* in the agarose gel is also introduced, which decreases the amount of DNA required for detection in the restriction fragment mapping by 30 to 100 fold compared with the ethidium bromide staining procedure.

(6) In following our original proposal, we succeeded in developing a 2-D gel electrophoresis technology to resolve at least 1,000 to 1,500 restriction enzyme generated fragments in an agarose gel. We planned to have a further improvement of the 2-D gel electrophoretic resolution.

We have designed several technological innovations to enhance the two-dimensional (2-D) electrophoretic size separation of DNA fragments within agarose gels. Improvements in resolution were obtained by a) using cylindrical rather than rectangular wells, b) non-parallel rather than parallel electrode orientations, c) planar, triangular, deformable electrodes of simple or complex design and d) large, rather than small, format gels. Also, by stacking two or more identically poured gels on top of each other, and running them simultaneously within a common electrophoresis chamber, it was possible to run a few or several gels under nearly identical conditions. These innovations, when used singularly or in combination, resulted in enhanced resolutions, savings in time, and/or improved reproducibilities when performing 2-D electrophoretic agarose gel separations of DNA fragments.

The conventional 2-D separation of fragments from large genomes is difficult. Band resolution is adversely affected due to crowding from a wide spectrum of fragment sizes. To address this problem cylindrical wells were used to compress the bands into compact spots instead of the elongated rectangular bands created by conventional well forming combs.

Lanes 1, 2 and 3 of figure 1 show λ Hind III DNA fragment bands as they appeared when different concentrations of total DNA were loaded into cylindrical wells. Lanes 4, 5 and 6 contain varying concentrations of fragments after size separation following entrance into the gel through rectangular wells.

Effects of Interelectrode Spacing and Electrode Surface Areas on the Mobility of DNA Fragments.

Electrophoresis is a complex process in which solvated anions and cations move at different rates and in opposite directions, driven by an electric current, within an electrolytic solution. According to Ohm's law, the electromotive force (E) is the product of the current (i) passing through a medium and the resistance (r) to the flow of charged particles created by the medium. The electric field strength is nominally defined as the electrical field per unit distance separating the anode and the cathode⁴. Assuming a homogeneous electrolytic medium and constant electromotive force, as the distance between two oppositely charged electrodes is increased the electrical field decreases. This is a direct consequence of an increase in the amount of medium, and thus the resistance, through which ions must pass through as they move towards their respective electrodes of attraction.

In one set of experiments the electrode surface area was varied between electrophoretic runs. In these cases the interelectrode distance and other

conditions (time, voltage, temperature, buffer composition) were held constant. For a fixed amount of electrophoretic separation time the DNA fragment migration distance increased with increasing electrode surface area. This effect was more pronounced for smaller fragments than for larger ones (see figure 2 A).

To study the effect of interelectrode distance on DNA fragment migration two electrodes, each possessing an electrolytically exposed surface area of 900 mm², were used. The electromotive force was kept constant at 20 volts during each electrophoretic separation. The time of each electrophoretic run was 5 hours. Figure 2 B is a graphical representation of the results of these experiments.

Under these conditions, the distance of migration for all fragments decreased with increasing interelectrode distance. The effect was less pronounced for the larger fragments than for the smaller sized ones.

Based on these results an electromotive force field gradient was generated across an agarose gel by placing normally parallel electrodes into non-parallel orientations.

Figure 3 A is a photograph of an EtBr stained agarose gel that contained 1 µg of λ Hind III DNA in every other lane. The DNA samples were size separated as described in the corresponding figure legend. The employed electrode geometry is shown in figure 3 B. Specifically, a linear platinum wire cathode was placed at a 24° angle relative to the gel's cathodic edge. The linear platinum wire anode was kept parallel to the gel's anodic edge.

From the photograph in figure 3 A, if an imaginary line were drawn connecting the 23,130 bp λ Hind III fragments from the left side of the gel to the right it be at an approximately 3° angle to an imaginary line drawn through these 23 kbp fragments had the DNA samples been exposed to a homogeneous electric field with an interelectrode working distance of 20 cm. This angle was named: the nonparallel electrode induced angle (N-PEI angle). The size of the N-PEI angle was larger for smaller fragments than for the larger ones. The N-PEI angle for the 2.3 kbp fragment was measured to be about 4.2°.

Electrophoresis of the samples shown in figure 3 A was performed for an additional 4 hours using the electrode configuration shown in figure 3 D. The results of this separation are shown in figure 3 C. In this case the N-PEI angles for the 23 kbp and 2.3 kbp fragments increased to 3.3° and 7.3°, respectively. For the gels shown in the photographs of figures 3 A and 3 C the electric field gradients increased from left to right across the gels.

Placing electrophoresis electrodes in orientations that are increasingly out of parallel with one another, within a common two-dimensional electrophoresis plane shared by the horizontal slab gel, increases the N-PEI angling effect. N-PEI angling was used to increase the amount of fragment resolving area beneath the diagonal of a 2-D agarose gel.

The dramatic effect on DNA fragment mobility created by an electric field gradient is shown in figure 4 A. Into each of fourteen lanes of an agarose gel was added 1 µg of λ Hind III DNA. A pair of nearly identically

shaped, curved, triangular, planar electrodes were positioned at angles to one another. The relative orientation of the electrodes is shown in figure 4 A by the dotted lines. Figure 4 B illustrates the general shape of the electrodes used in this electrophoretic separation. The anomalous running of the 23 kbp fragments near the top left quadrant of the photograph arose from poor buffer recirculation in the cathodic reservoir.

Method for Predicting Sizes of Fragments Resolved By 2-D Agarose Gel Electrophoresis with Nonparallel Electrode Geometries.

When conventional agarose gel electrophoresis is used size marker DNA fragments are generally loaded in lanes parallel to those containing sample DNA. Sizes of unknown fragments are estimated by comparing their mobilities with those of known sized fragments. When DNA samples are size separated through a nonhomogeneous field the conventional approach for fragment size estimation becomes inadequate. To overcome this problem a method of running two gels simultaneously within the same electrophoresis tank has been developed. This was necessary so as to allow DNA size markers to be size separated simultaneously in one gel while the test 2-D DNA samples are separated in a second gel. With this arrangement the two gels would experience essentially identical running conditions.

Figures 5 A and 5 B are photographs of EtBr stained agarose gels containing a dilution series of λ Hind III DNA fragments. The gel shown in A was positioned directly on top of the gel shown in B. After DNA samples were loaded into the wells of the two gels conventional agarose gel electrophoresis, using parallel wire electrodes, was performed. The results of a densitometric analysis of the DNA size separated in lane 1 of the top and bottom gels are shown graphically in figure 5 C. Each of the different sized DNA fragments migrated to nearly the same extent whether present either in the top or bottom gel.

These results indicate that it is possible to simultaneously prepare a 'size-marker' grid. This grid would be used to estimate sizes of unknown fragments in test and control 2-D agarose gels. Size marker grids would be generated by running the size marker containing gel stacked either above or below the 2-D gel containing DNA samples of interest.

Large Format Agarose Gel Electrophoresis.

The resolving capabilities of 2-D agarose gel electrophoresis was increased dramatically by resorting to large sized agarose gels.

An oversized electrophoresis tank was built to accommodate a 50 cm X 40 cm agarose gel. The basic dimensions of the large format agarose gel equipment is depicted in figure 6. Gels prepared for this equipment would have resolvable 2-D gel electrophoresis areas approximately 4X greater than gels prepared in commercially available (BRL, Gaithersburg, MD) 20 cm X 25 cm format size.

2. 3-Dimensional Gel Electrophoresis.

With all the experiences and improvements, at best we can separate 3,000 fragments, as well defined autoradiographic spots, in a 2-D gel

electrophoresis experiment. We have utilized this technological advancement by using an interspersed, repetitive DNA sequence, as probe, which has been described earlier.

We are in the final stage of the development of a 3-D gel electrophoresis procedure in which the DNA fragments in the 2-D gel plane are forced to pass through a planar, affinity trapping layer. Through this layer the desired DNA fragments can be trapped and isolated or alternatively through this layer all the unwanted DNA fragments can be trapped with only the desired DNA fragments passing through and onto a catching membrane.

The DNA Difference Detection (DDI) Approach.

We have begun developing a procedure to search for sequence differences existing between two syntenic genomic DNA samples. A flow diagram of the scheme is shown in figure 7.

The procedure involves complete restriction enzyme digestion of the two samples, end-labeling one genomic sample (Target) with radioactive nucleotides and biotinylating the other sample (Driver) with biotinylated nucleotide analogs. The samples are mixed in a 1 to 1,000 ratio and size separated in the first dimension through an agarose gel. *In situ* digestion with a second restriction enzyme is performed on the size separated DNA. The DNA is then size separated in the second dimension using 2-D techniques described earlier in this report and in appropriately referenced articles published out of this laboratory. In-gel denaturation and renaturation is then performed *in situ*. This has the effect of binding unrearranged radioactively labeled fragments to unrearranged biotinylated complementary strands. Rearranged radioactive fragments would be located in different positions of the 2-D gel slab and would not be able to reanneal with a biotinylated complement. In the next step the DNA molecules are electrophoresed out of the 2-D plane, through a streptavidin-agarose trapping gel and onto a DEAE-cellulose sheet. The DEAE-cellulose membrane is then subjected to autoradiography to determine the number and locations of fragments that are rearranged in the two samples.

The technically challenging portions of this scheme involve a) determining the proper in-gel denaturation and reannealing conditions b) designing a workable electrophoresis chamber to perform the third-dimensional separation and c) developing the Streptavidin-agarose gel trapping technology.

We are currently in the process of determining the proper reannealing conditions to use once the size separated DNA fragments, in the 2-D gel, are alkali denatured. The technological difficulties are several. First, the reannealing process must occur sufficiently slow and in the proper salty milieu to prevent fragment aggregation within the gel. Secondly, the conditions should not encourage reannealing of complementarily unrelated fragments to one another and thirdly, the reannealing process should not take more than a few dozen hours, or several days at the most, for the Cot requirements to be met. We plan to test reannealing under various low salt, high temperature and formamide concentrations for different time intervals. We will check for proper reannealing by using an appropriate model system. Such a system is described below.

To date we have constructed and tested a workable 3-D electrophoresis

tank called the electrotransfer chamber. This custom built device evolved from prototypes that were designed around graphite or aluminum planar electrodes and another prototype that used platinum wire grid electrodes. We have found that perforated stainless steel plates provide the best and most consistent results of all the electrodes that we have tested. The stainless steel electrodes are relatively inexpensive to produce, can be obtained in sizes that meet our needs, and do not undergo perceptible electrolytic degradation during the course of a 3-D electrophoresis run.

Another challenge, in constructing the electrotransfer chamber was the design of a suitable frame that would hold the 2-D composite gel in the proper location and orientation in the tank without gel collapse or interference with the electro-phoretic current field. We have designed and built a final prototype that meets all of these requirements. The current frame uses an acrylic outer frame with cotton gauze fabric spanning the gel supporting area. The cotton gauze is easily wetted by the electrophoresis buffer, allows the current to move from electrode to electrode without detectable interference, does not trap gas bubbles on its fibers (as does polypropylene fabric) and is sufficiently taut to support the gel.

Figure 8 a) depicts an exploded view of the composite 2-D gel in relation to the two stainless steel electrodes. Diagrams 8b and 8c are top and side views, respectively, of the electroblotting chamber.

Development of the Streptavidin-agarose trapping gel has been completed. We have determined how to create a molten agarose liquid that contains unmelted agarose beads that have streptavidin molecules covalently bound to them. This process does not inactivate the streptavidin. It is conducted in such a manner so that once the gel solidifies the bound streptavidin is evenly dispersed throughout the trapping gel and is completely active with regard to its ability to bind biotinylated DNA fragments. Figure 9 shows results of a preliminary trapping experiment.

Once the proper conditions have been elucidated we will be prepared to test the DDI procedure from beginning to end on a test system. The test system would contain *E. coli* genomic DNA as the Driver DNA and *E. coli* genomic DNA with single genome equivalent of lambda DNA to serve as the Target DNA sample. The Target sample, in this case, would mimic the case of a syntenic genome that has an insertion that is absent in the Driver genome.

CONCLUSIONS

Cylindrical wells cause fragment bands to migrate in both the 1st and the 2nd dimensions as compact, circular spots. Since the DNA fragments are concentrated into spots of smaller area, than those originating from conventional rectangular wells, less sample is needed per lane to detect the fragments.

The use of stacked gels provide the advantages of i) reduced electrophoresis time (i.e. 2 or more gels can be run simultaneously in the same apparatus), and ii) reproducibility of gel running conditions and results between gels. Disadvantages of using stacked gels are i) number of gels that can be stacked per electrophoresis tank is limited by the thickness of the gels, ii) improper electrode placement may result in different gels being

exposed to different electric field strengths, and iii) gels that are not properly leveled may have their DNA fragments migrate out of the gel at oblique angles (data not shown).

Nonparallel oriented, curved, triangular electrodes, generates electric field gradients. These gradients can be used to decrease the angle of the 2-D diagonal and thus increase the amount of agarose gel area used for resolving DNA fragments.

Large format agarose gels having agarose bed areas of 2,000 cm² can increase the resolvable gel area to approximately 4X or more relative to most commercially available gel electrophoresis apparatus.

An improvement in resolution of at least 8-fold should be obtained by combining the discussed improvements to 2-D agarose gel electrophoresis into each electrophoretic separation procedure performed. But data analysis may be much more difficult with N-PEI because the fragments migrate non-linearly through the gel. To address this problem the simultaneous use of a size marker gel, stacked on the sample gel, is recommended. This stacked gel approach is a method for producing a size marker grid for determining sample fragment sizes.

Application of the 2-D electrophoresis Southern blot method to compare the human placental DNA of two individuals provided good resolution of approximately 900 fragment bands and the revelation of 15 polymorphic fragment signals. The use of the Diagonal Line Fragment Resolution procedure has allowed us to compare the two genomes in a more thorough manner by increasing the overall resolution of 2-D agarose gel size separated restriction fragments.

Finally, the use of the DDI procedure, once it is fully developed and tested, would be useful to study the degree of global fluidity of the human genome without relying on specific gene probes to do so. The extent of human genome fluidity is currently unknown. How fluid it is remains a fundamentally important question to answer in light of current efforts and future undertakings of the Human Genome Initiative to obtain the complete primary sequence of the human genome.

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Figure 1 Cylindrical sample receiving wells versus rectangular sample receiving wells for agarose gel electrophoresis. Lanes 1 and 6 contained 1.0 μ g of λ Hind III DNA, lanes 2 and 5 contained 0.5 μ g and lanes 3 and 4 contained 0.1 μ g each. The DNA samples were size separated through 0.8% agarose gels with 1X TAAE buffer at 1.5 v/cm and 22° C for 3½ hours. The gels were stained with EtBr and photographed using u.v. trans-illumination.

Figure 2 Effect of A) electrode surface area and B) interelectrode distance on DNA fragment mobility.

Figure 2B)

Figure 3   Effect of nonparallel electrode orientations on the mobilities of DNA fragments.

A) 1% agarose gel containing 1 μ g of λ Hind III DNA in every other lane. Electrophoresis was for 6 hours at 1.5 v/cm in 1X TANAE buffer and 0.5 μ g/ml EtBr and room temperature. The cathodic electrode was positioned at 24° relative to the cathodic edge of the gel. C) Same gel shown in A after altering electrode to gel angles of both electrodes and electrophoresing the fragments for an additional 4 hours under identical conditions as for A. B) and D) Diagrammatic depictions (not drawn to scale) of the electrode configurations, with pertinent sizes and angle measurements, of equipment used to generate the separations shown in A and C, respectively.

C)

D)

A)

B)

Figure 4 A) Agarose gel electrophoretic size separation of DNA fragments using curved, triangular electrodes. B) Schematic diagram representing the approximate shape (side view) of the anodic and cathodic electrodes used to perform the electrophoretic DNA fragment separation shown in A. Dotted lines (-----) represent relative positions of electrodes. (+) represents anodic electrode and (-) represents cathodic electrode. Electrophoresis conditions: 0.8% agarose gel, 1X TANAE buffer, 1.5 v/cm, 22°C, for 4 hours.

Figure 5   Stacked agarose gel electrophoresis. Photograph of u.v transilluminated, EtBr stained agarose gels following size separation of λ *Hind* III DNA fragments through a gel situated A) on top ( ) of or B) below ( ) the other. C) Bar chart comparing the relative distance of migration of the fragments present in lane 1 of the top gel and the bottom gel . Lanes 1 and 7 were loaded with 1.0 μ g of λ *Hind* III DNA while lanes 2, 3, 4, 5, and 6 contained 0.5 μ g, 0.1 μ g, 0.05 μ g, 0.01 μ g and 0.005 μ g of total λ *Hind* III DNA, respectively.

Figure 6 Schematic representation of the large format, horizontal, agarose slab gel electrophoresis tank.

$$\text{Gel size} = (50 \text{ cm} \times 40 \text{ cm}) = 2000 \text{ cm}^2$$

Difference DNA Detection

Figure 7

Figure 8a

Exploded view of electrotransfer cassette, situated between the electrode plates, in relation to the 2-D gel, the streptavidin-agarose trapping gel and the DEAE-Cellulose membrane.

8b

8c

Figures 8b and 8c

Figure 9. Streptavidin-agarose trapping. Pictured is an autoradiogram of a gel following electrophoresis of biotinylated and unbiotinylated DNA either through Streptavidin-agarose trapping gel (lanes 1 and 2) or not (lanes 3 and 4). All lanes contained 20 ng of radioactively labeled lambda DNA that had been previously digested to completion with *Hind* III. A streptavidin-agarose gel was poured in a trough 0.5 cm from lanes 1 and 2 and spanning these lanes for 0.5 cm. The DNA in lanes 2 and 4 were biotinylated and the DNA samples in lanes 1 and 3 were not biotinylated. Results: The unbiotinylated DNA in lane 1 migrated through the Streptavidin agarose gel strip to the same extent as unbiotinylated DNA (lane 3) that did not pass through the Streptavidin-agarose gel. This indicates that unbiotinylated DNA was not retarded by the Streptavidin-agarose beads. Also, The biotinylated DNA in lane 2 was trapped when it entered the Streptavidin-agarose gel strip as evidenced by the dark autoradiographic spot near the well of lane 2. The biotinylated DNA in lane 4, which did not pass through a Streptavidin-agarose gel strip, migrated to the same extent as unbiotinylated fragments indicating that the presence of biotin moieties on the fragments did not appreciably retard the modified DNA fragments.

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APPENDIX

1. Callahan, D.E., Karim, A., Zheng, G., Ts'o, P.O.P., and Lesko, S.A. Quantitation and mapping of integrated human papillomavirus on human metaphase chromosomes using a fluorescence microscope imaging system. *Cytometry* 13:4530461 (1992).
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