On the possibility of obtaining a physical map of genomes by photoelectron imaging

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ABSTRACT Photoelectron imaging provides the possibility of a new method of mapping chromosomes. The basic concept is to cause DNA to emit electrons under the action of UV light. The criteria which must be met to map genomes by photoelectron imaging are set forth and discussed. Forming an image of the DNA by accelerating and focusing the electrons is a necessary but not sufficient condition for genome mapping. Equally important is to identify wavelengths of UV light which will cause selective emission from the base pairs, adenine-thymine and guanine-cytosine. The resulting image would then contain a modulation in the image brightness along the DNA duplex. By examining the photoelectron current from uniform films of homopolymers, a wavelength region is identified where marked differences in emission from base pairs is observed. At 160 nm, for example, the relative electron emission from a film of poly(dGdC) is ~5 times greater than for an equivalent film of poly(dAdT). Using the experimental data and known sequences, photoelectron gene maps are calculated for the bacteriophage lambda and for a short interspersed repetitive DNA sequence (an Alu repeat) of the human genome. The results suggest that a 5-nm physical map of chromosomes generated by photoelectron imaging would be informative and useful in mapping human and other large genomes.

INTRODUCTION

The ultimate physical map of a chromosome is the sequence of bases of the DNA. However, mapping of a genome at several levels is useful. Much information is being obtained, for example, even at the modest resolution of the light microscope. Observing the banding patterns in dye-stained metaphase chromosomes in the optical microscope has provided a framework for developing a physical map of human chromosomes. When combined with techniques such as somatic cell hybridization and in situ hybridization, optical microscopy of chromosomes has made possible the identification of many genes on chromosomes, and of abnormal duplications and translocations linked to genetic diseases (Macgregor and Varley, 1983; Yunis, 1983). Increasing the resolution of these physical maps by transmission electron microscopy (TEM) and scanning electron microscopy has been frustrating, due in part to the condensed structure of metaphase chromosomes. An alternative approach is to focus on well-spread duplex DNA. Early successes in electron microscopy of DNA include autoradiography of radiolabeled DNA (Cairns, 1963) and denaturation mapping (Inman, 1966). These techniques have provided important insights into mechanisms of DNA replication (Kornberg, 1974) and continue to be useful. Subsequent advances in chromosome mapping using conventional electron microscopes have been slow in coming, primarily because electron scattering provides little or no contrast between the DNA bases, and contrast enhancement by means of heavy atom labeling is difficult to achieve. However, some progress in TEM is being made with colloidal gold labeling (Hutchison et al., 1982; Hiriyanna et al., 1988).

We propose here an alternative approach to the mapping of chromosomes. The physical principle is the photoelectric effect. When matter of any kind is subjected to photons of sufficient energy, electrons are ejected from the surface. These electrons can be simply collected as in photoemission current measurements, energy analyzed by photoelectron spectroscopy, or used to form an image of the object in an instrument known as a photoelectron microscope or photoemission electron microscope (for a review see Griffith and Rempfer, 1985; Griffith and Rempfer, 1987). Photoelectron microscopy in physics dates back to the earliest days of the development of electron microscopy in Germany (Brüche 1933; Pohl, 1934) and biological applications date from 1972 (Griffith et al., 1972). The first images of partially condensed DNA were reported in 1983 (Houle and Griffith, 1983). However, the high resolution imaging of duplex DNA is a new development (Griffith et al., 1990), and the present proposal rests, in part, on this technical advance. Also relevant is the literature on quantum yields and photoelectron spectroscopy of DNA and nucleic acid bases. The early work on solid films (Berger, 1968; Pong and Inouye, 1976; Sukhov et al., 1976) and the higher resolution gas phase studies of purines and pyrimidines by Dougherty

and McGlynn (1977); and by LeBreton's group (Yu et al., 1978; Urano et al., 1989) indicate there are differences in the ionization potentials of the nucleic acid bases. Here, we report some additional observations on the photoelectric effect of DNA base pairs and use this information to model the proposed photoelectron gene mapping experiments. The results indicate that photoelectron imaging of well-spread DNA, combined with digital image processing, has the potential of providing a high-resolution physical map of genomes.

MATERIALS AND METHODS

Chemicals

Poly(dGdC), an alternating double-stranded polymer containing repeating GC units, was obtained from Pharmacia Fine Chemicals (Piscataway, NJ) and Boehringer-Mannheim Diagnostics, Inc. (Indianapolis, IN). Poly(dAdT) was obtained from Pharmacia Fine Chemicals, Boehringer-Mannheim Diagnostics, Inc., and Midland Certified Reagent Co. (Midland, TX). The polymers, which are sold lyophilized as the sodium salts, were dissolved in glass distilled water at 200 μg/ml and dialyzed extensively against distilled water before use to remove any excess salts that could deposit on the films.

Specimen preparation

The sample mounts used for photoemission measurements were 5 mm diam round glass coverslips (Bellco Biotechnology, Vineland, NJ), which were made conductive by a thin layer (~5 nm thick) of vacuum evaporated chromium. Before use the coverslips were cleaned by bath sonication in an aqueous solution containing 3 M HCl and 3% H_2O_2, and washed with glass distilled water. The coverslips were then floated on a drop of a 1-mg/ml aqueous solution of dextran derivatized with spermine (Mrsny et al., 1987). The spermine-derivatized dextran treatment makes the hydrophobic chromium surface hydrophilic and provides a positively charged surface to facilitate binding to the polynucleotides. The derivatized dextran-treated coverslips were washed extensively with glass distilled water, and after the last rinse, almost all of the water was drained off. While the coverslips were still wet, 20 μL drops of polynucleotide solution were added and allowed to dry forming solid films of polynucleotide. From the amount of polynucleotide placed on the coverslips the thickness of the films was estimated to be on the order of 200 nm. The quality of the films was checked by light microscopy. The molecular weight distributions of the polynucleotides used differed, and some film quality variations were observed. In these experiments the Pharmacia preparations gave the highest quality films and were used for the data reported here. Measurements obtained with the other commercial preparations, however, were consistent with data from the Pharmacia samples.

Photoemission measurements

An oil-free, ultra-high vacuum photoelectron microscope described previously was used (Griffith and Rempfer, 1987). In its normal operating mode, the microscope is equipped with two short-arc mercury lamps to stimulate photoemission. For these experiments one of the mercury lamps was removed and replaced with a 0.3-m scanning vacuum monochromator (McPherson, Division S.I. Corp. Acton., MA) attached to a high brightness deuterium lamp (model L2196; Hamamatsu Corp., Middlesex, NJ) with a MgF_2 window. The induced photoelectron beam current was measured as a function of wavelength with a picoammeter (model 26000; Keithley Instruments, Inc., Cleveland, OH) interfaced to a Dascon-1 A/D data acquisition card (Metrabyte Corp., Taunton, MA) in an AT computer (IBM Instruments, Inc., Danbury, CT).

Calculation of photoelectron gene maps

Nucleic acid base sequences for bacteriophage lambda (Lambda-clindlts857Sam7; Sanger et al., 1982) and for human Alu family, clone BLUR7 (human placental DNA, HUMRSAB7; Deininger et al., 1981) were obtained from the GenBank genetic sequence data bank (Bilofsky et al., 1986). Tabled frequencies of patterns of A (or T) and G (or C) were made as fractions of a "window" of N base pairs (generally N = 15) along the entire genome lengths. The window was selected to match an expected instrument resolution of 5 nm and was translated along the sequence, shifting 1 base pair before averaging for each calculation. In the actual experiment, the specimen is not scanned but rather electrons are collected from the entire specimen. Nevertheless the result would be a modulation of brightness along the DNA strand that corresponds to a running average of this type. The resulting data files were then read and the experimentally derived emission ratio (GC/AT = 5.5) was applied to the frequency distribution of the paired bases. The results were plotted as the base pair number versus the photoelectron emission expected at each 15 base pair window's center. No curve fitting is done through the data points. In an experimental emission plot there would be a natural smoothing of the data. Experimental tests of these plots must await a redesign of the photoelectron microscope to optimize the amount of vacuum UV light reaching the specimen (the present instrument was designed for UV rather than vacuum UV excitation).

RESULTS

Photoelectron emission

Measurements of the total photoemission current as a function of wavelength were made for thin films of the copolymers poly(dGdC) and poly(dAdT). Representative results of these photoemission current measurements are given in Fig. 1. There was some variation in photoemission data which can be ascribed to variations in film quality. All sets of data, however, gave essentially the same result: the emission current from the poly(dGdC) films is much greater than the emission current from the poly(dAdT) films. The ratio of emission currents from Fig. 1 is GC/AT = 5.5 at the poly(dGdC) maximum near 160 nm.

Care must be taken not to over interpret these data. A deuterium lamp was used to stimulate photoelectron emission and the data are uncorrected for variations in lamp intensity with wavelength. A family of intense sharp lines exists around 160 nm (Samson, 1967). In these experiments the slits of the vacuum monochromator were opened so that the lamp output was broadened into a continuous peak. Thus, the maximum in Fig. 1 is a

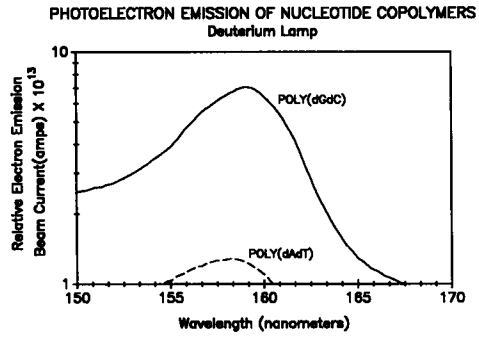

FIGURE 1 Experimental plots of electron emission from thin films of poly(dGdC) and poly(dAdT) versus wavelength of the exciting light demonstrate that large differences in the photoelectric response occur in the far UV. The emission is not corrected for the variation of deuterium lamp output with wavelength.

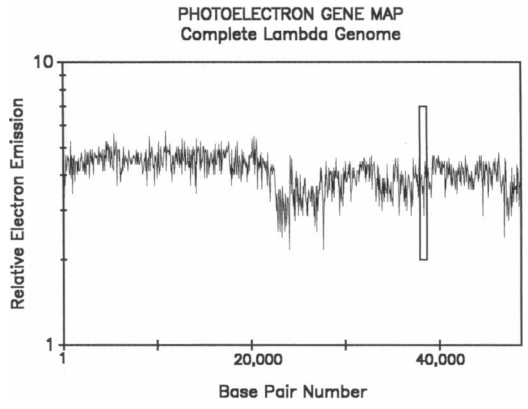

FIGURE 2 The photoelectron fingerprint calculated for the complete bacteriophage lambda sequence reflects the GC content of the genome. This plot was generated by weighting the known sequence of lambda with the contrast ratio of GC/AT = 5.5 taken from Fig. 1. The plot is a window representing a moving average of 50 base pairs along the length of the molecule. The box is an arbitrary small segment of the genome which is enlarged and replotted in Fig. 3.

property of the deuterium lamp, and does not mean that there is a maximum at this point in the photoelectron quantum yield curves. It is the relative positions of the two curves that is significant. The cutoff at the bottom of Fig. 1 at a beam current of 1×10^{-13} A simply represents the limit of sensitivity of the detection system.

Some data were obtained at longer wavelengths (not shown). Using the 185-nm line of a low pressure mercury lamp the emission from the films of poly(dGdC) was observed to be greater than films of poly(dAdT). A high pressure mercury lamp (model HBO-100; Osram Corp., Newburgh, NY) with a presumed short wavelength cutoff of 254 nm also gave the result that the emission from poly(dGdC) was greater than from poly(dAdT). However, as the wavelengths are increased, the emission of both polymers decreases. This limits the usefulness of longer wavelengths in high resolution photoelectron imaging unless double quantum transitions are involved.

Calculated photoelectron gene maps

Knowing the relative emission from poly(dGdC) to poly(dAdT) allows one to simulate the corresponding variation in brightness that should occur along the image of the DNA duplex in high resolution micrographs. The question we are aksing here is: Given the resolution of a photoelectron microscope, is the information content of the brightness modulation sufficient to produce a useful fingerprint of the DNA? For convenience we refer to these plots as photoelectron gene maps or photoelectron fingerprints. Fig. 2 is a photoelectron gene map of the entire bacteriophage lambda genome, consisting of 48,502 base pairs. Fig. 2 necessarily resembles a plot of the GC content of the genome (see Sanger et al., 1982). At this low resolution the asymmetry in left and right halves of the genome is clearly evident, but it is not obvious that the fine structure is information rather than noise. Fig. 3 is an expanded plot of the section of DNA in the small square of Fig. 2. This section was chosen simply because it contains parts of the familiar lysogenic/lytic genetic switch. Fig. 4 is similar simulation for a common repeat of the human genome, an Alu repeat. This approach can be extended to single-stranded DNA and RNA. The information content would be greater. However, imaging single strands of nucleic acids would be difficult and this is not the normal state of genomic DNA.

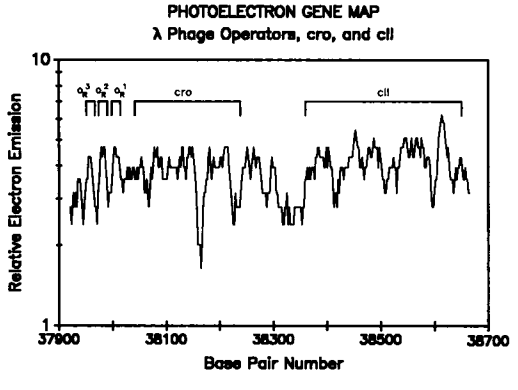

FIGURE 3 An expanded segment of the photoelectron fingerprint of bacteriophage lambda of Fig. 2 demonstrates that known features are recognizable at 5 nm resolution. Plotted with a moving average of 15 base pairs, the graph details the part of the genome that controls the early stage of gene expression in lambda. Indicated are the three operators (O_R^1, O_R^2, O_R^3), the cro gene and the cII gene.

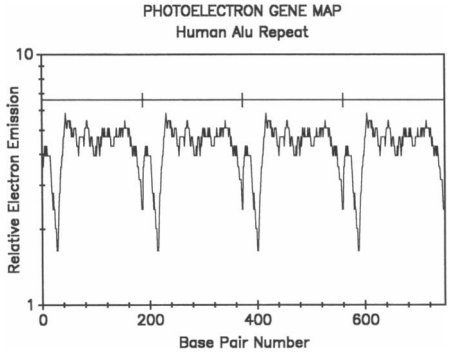

FIGURE 4 A photoelectron fingerprint calculated for the human Alu family BLUR 7 repeat shows distinctive features that may prove useful as a marker on the human genome. The 201 base pair segment is repeated here four times without intervening sequences to save space. However, on the human genome it occurs as an interspersed repeat rather than a tandem repeat. This plot was generated with a contrast ratio of GC/AT = 5.5 and a window representing a moving average of 15 base pairs.

For these reasons, we focus on duplex DNA in the present study.

DISCUSSION

Photoelectron imaging has the potential of providing a new type of physical map of genomes. The experiment is reminiscent of autoradiography because the DNA is the source of electrons. There are major differences, however. The kinetic energy of electrons in photoelectron imaging is much lower, on the order of 1 eV or less, which makes it possible to image them directly (rather than developing silver grains and observing these by TEM, as is done in autoradiography). Also, the number of electrons emitted can be much greater in photoelectron imaging than in autoradiography, and the same molecule may emit electrons more than once (by replacing emitted electrons with electrons from the cathode power supply). Photoelectron imaging used DNA very nearly in its native state, i.e., DNA which has not been labeled, fixed, or stained.

An important feature of photoelectron imaging is that it is a selective process. Each molecule has a unique electronic structure and hence a unique set of ionization potentials. If the wavelength of light is gradually shortened, the photoemission of molecules will be "turned on" at different wavelengths, the one having the lowest ionization potential becoming visible first. This is similar in some respects to fluorescence microscopy where the emission of dye molecules can be controlled by selective excitation.

To utilize this basic physical principle (e.g., the photoelectric effect) in a practical method of mapping genomes, several conditions must be met. These are listed in Table 1. The first condition is instrumental and has been largely achieved. The practical resolution of the present photoelectron microscope is 10 nm or better, and efforts are underway to achieve the theoretical limit of 5 nm (Rempfer and Griffith, 1989). This is sufficient for the level of genome mapping proposed here. Looking toward the future, it is possible, in principle, to further increase resolution by correcting the chromatic and spherical aberrations in the imaging process (Rempfer and Griffith, 1989). The ultimate limit, imposed by diffraction, would then approach 1 nm for 1 eV electrons.

The second condition of Table 1 is contrast. In this study we set out to find if there exists a wavelength region in which the photoemission for GC base pairs is much greater than for AT base pairs. Fig. 1 demonstrates that such a region does exist. The differences in quantum yields are caused by differences in the electronic structure of these molecules and base pairing may be a contributing factor. These data also indicate that photoionization from lone pairs of electrons on the deoxyribose or phosphate groups, if any, does not mask the photoemission from the base pairs. The contrast difference is sufficient for photoelectron gene mapping, as demonstrated in the simulations of Figs. 2–4. Fortunately, the region of 160 nm also corresponds to a fairly intense band of the deuterium lamp, which may satisfy criteria 3 (Table 1), the availability of sufficient light for high magnification photoelectron microscopy of DNA. Continuous wave (cw) lasers within the wavelength range 150–240 nm would have obvious advantages. Synchrotron radiation is another possibility for some laboratories. Pulsed lasers

TABLE 1 Criteria to be met for mapping genomes by photoelectron imaging

(1)	The photoelectron microscope must have sufficient resolution to image DNA duplexes without shadowing, staining, or labeling.
(2)	There must exist a wavelength region in which photoemission of one base or base pair is significantly greater than the others.
(3)	An intense source of exciting light in this wavelength region must be available which can be used for high magnification experiments.
(4)	The substrate must be flat and less photoemissive than either of the base pairs.
(5)	The DNA must be uniformly spread on the substrate, with a minimum of overlapping regions.
(6)	Any photochemistry occurring in the specimen or substrate must preserve a contrast difference between base pairs, and between the DNA and substrate

having high peak powers can be ruled out because they produce space charge effects which distort the images (Massey et al., 1981).

The remaining three conditions of Table 1 are self-explanatory. It would be naive, for example, to assume that photochemistry does not take place in the far UV. However, the only criteria that must be met is that any photochemistry of the DNA bases be sufficiently different to maintain a measure of contrast. This has yet to be tested. Specimen damage occurs to some extent in all types of microscopy. Damage is likely to be much less in photoelectron imaging than conventional electron microscopy because there is no beam of electrons impinging on the specimen.

An area of technical development required for the success of photoelectron gene mapping is image analysis. Computer processing of the micrograph negatives will be needed to locate the image of each DNA strand on the photoelectron micrographs and linearize the information for plotting in the form of Figs. 2–4. Another program will be required to search and recognize features of the photoelectron fingerprints. We note that algorithms are under development for other applications that might be modified for these tasks (e.g., Castleman, 1979, Nazif and Levine, 1984; Zhu and Kim, 1989; Nederlof, M. A., personal communication).

To gain a more detailed understanding of the photoemission process, it is useful to consider it as a four step process: (*a*) absorption of a quantum of UV light at a distance *d* below the surface, (*b*) photoionization, (*c*) transport of the electron to the surface, and (*d*) escape into the vacuum. The overall efficiency or quantum yield (*Y*, the ratio of emitted electrons/incident quanta) is a product of the yields for the four individual processes. The escape depth, *d*, is fairly short because the kinetic energy of the emerging photoelectrons is small (i.e., 1 eV). Typical escape depths of organic molecules are on the order of 0.5–5 nm (Houle et al., 1982). Because duplex DNA is ~2 nm in diam (B form), the electrons can easily escape from the partially buried base pairs into the vacuum, regardless of the orientation of the helix on the substrate.

The contrast ratios reported here are consistent with the photoemission literature, although detailed comparisons are difficult because the experimental conditions vary. The most accurate data in the literature are the gas phase ionization potentials (I_g). Urano et al. (1989) report lowest gas phase ionization potentials of 8.48 eV for adenine, 9.18 eV for thymine, 8.09 eV for 1,9-dimethylguanine, and 8.65 eV for methyl cytosine (methyl derivatives of cytosine and guanine were used to avoid problems associated with the occurrence of more than one tautomeric form in the gas phase). Thus, guanine has the lowest ionization potential of the four DNA bases, and the average for G + C is below that of A + T. The ionization energy (photoemission threshold, I_s) of solid organic compounds is lower than the gas phase value by ~1.5 eV due to polarization effects (Gutmann and Lyons, 1967). This would suggest an emission threshold of 6.6 eV for guanine, or ~190 nm. One must work below threshold to assure a sufficient quantum yield (*Y*), so 160 nm (i.e., Fig. 1) would appear a reasonable starting point. If photochemical damage is too great at 160 nm, longer wavelengths may be explored.

We stress that Figs. 2–4 are theoretical predictions, not experimental gene maps. They do, however, provide useful insight into the information content of photoelectron images of DNA. Furthermore, these graphs are realistic in that they utilize the experimental contrast ratio of Fig. 1 and are plotted to a scale that corresponds to the resolution of current photoelectron microscopes. We do not expect photoelectron imaging to develop into a method of sequencing DNA. The goal is to develop a method that would bridge the gap between optical micrographs of metaphase chromosomes and the DNA sequence. At the present resolution, plots are fingerprints which would contain considerable detail about the genome. For example, in Fig. 3, the three operators (O_R^3, O_R^2, O_R^1) which bind the Cro and lambda repressor proteins show up as individual peaks even though each of these operators is only 17 base pairs in length.

One third of the human genome is composed of a variety of repeated DNAs (Britten and Kohne, 1968). One of these repeats (Alu) was selected to test whether the features would be sufficiently distinctive to act as a natural "molecular banding pattern" that might be recognizable throughout the genome. The results are quite encouraging in this respect (see Fig. 4). Because the genome is linear, it is not difficult to envision a computer pattern recognition program capable of recognizing this repeated motif. The large amount of repetitive DNA would be an important asset rather than a hindrance in this approach to the physical mapping of chromosomes.

In summary, a new method of generating a physical map of genomes based on the photoelectric effect is proposed. The following steps have been taken toward translating this idea into a practical genome mapping technique: (*a*) The instrumentation (photoelectron microscopy and image processing) has developed to the point where well-spread duplex DNA preparations can be imaged. No staining or shadowing or labeling is required. (*b*) A UV excitation region has been identified in which there is marked contrast between guanine-cytosine and adenine-thymine base pairs. (*c*) Photoelectron gene maps based on known sequences and the experimentally observed contrast ratios have been calculated. These

simulations show sufficient detail to provide a unique fingerprint at the theoretical limit of current photoelectron microscope resolution (5 nm). There are several potential advantages of photoelectron gene mapping: First, it would be a rapid method of mapping large genomes. Data is collected in parallel from all parts of the specimen rather than sequentially, as in scanning techniques. The total collection time for one field of view (e.g., 1–100 μm^2) is <1 min. Second, no radioactivity is involved. Third, this method is sensitive because only a small number of copies of the DNA would be needed for analysis. Fourth, there is a simple relationship between sequence and a photoelectron gene map. Any known sequence could, in principle, be tested against a computer file of experimental plots similar to Figs. 2–4 to find its location on the plot. Finally, this technique could be used in conjunction with established methods such as in situ hybridization and restriction mapping. It may, therefore, prove to be useful in ordering libraries of large fragments of chromosomes and in comparative studies of genomes.

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