Genomic Sequencing

George M. Church, Walter Gilbert

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(DNA methylation/UV crosslinking/filter hybridization/immunoglobulin genes)

GEORGE M. CHURCH* AND WALTER GILBERT*†

*Biological Laboratories, Harvard University, Cambridge, MA 02138; and †Biogen, Inc., 14 Cambridge Center, Cambridge, MA 02142

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ABSTRACT **Unique DNA sequences can be determined directly from mouse genomic DNA. A denaturing gel separates by size mixtures of unlabeled DNA fragments from complete restriction and partial chemical cleavages of the entire genome. These lanes of DNA are transferred and UV-crosslinked to nylon membranes. Hybridization with a short ^{32}P-labeled single-stranded probe produces the image of a DNA sequence "ladder" extending from the 3′ or 5′ end of one restriction site in the genome. Numerous different sequences can be obtained from a single membrane by reprobing. Each band in these sequences represents 3 fg of DNA complementary to the probe. Sequence data from mouse immunoglobulin heavy chain genes from several cell types are presented. The genomic sequencing procedures are applicable to the analysis of genetic polymorphisms, DNA methylation at deoxycytidines, and nucleic acid–protein interactions at single nucleotide resolution.**

How can we visualize the state of individual nucleotides within large chromosomes? During recombinant DNA cloning, information about DNA methylation and chromatin structure is lost. Direct chemical modification of the genome combined with complete restriction enzyme digestion and separation by size on a denaturing gel preserves some of this information in the form of numerous comigrating sets of DNA sequence "ladders." To access one sequence at a time, the lanes of DNA are transferred and crosslinked to a nylon membrane and hybridized to a short single-stranded ^{32}P-labeled probe specific for one end of one restriction fragment within the genome. Fig. 1 illustrates why this works. The probe called "3′ lower" will hybridize to only three classes of DNA reaction products: the appropriate fragments extending from the 3′ end of the lower strand of the restriction fragment, the longest fragments from the 5′ end of the upper strand (which will only affect the top of the sequence ladder), and the middle fragments with both ends produced by chemical cleavage ("zigzags"). The abundance of the appropriate fragments is proportional to the probability (P) that the chemical reaction cleaves at any given target. Because the abundance of the middle fragments is proportional to P^2, interference can be diminished by decreasing the extent of the reaction. About one cleavage every 500 nucleotides is optimal. Decreasing the length of the probe also alleviates the problem of hybridization to fragments from the wrong end and the middle; however, very short probes (less than 20 nucleotides long) and low stringency washes can produce a crossreacting background. Probes 100 to 200 nucleotides long work well.

DNA Methylation. Up to 12% of all cytosines in vertebrate genomes are methylated mainly at C-G sequences. In plants, up to 50% of all cytosines are methylated mainly at C-G and C-N-G sequences (reviewed in ref. 1). Only a small subset of these methylation sites can be assayed by restriction analyses (2), and flanking sequences can severely affect the relative efficiencies of restriction enzyme recognition (3, 4). Transfection of *in vitro* methylated DNA into mammalian cells (5, 6) can demonstrate sites of cytosine methylation that affect RNA transcription, as long as the maintenance of the methylation at such sites can be followed. The ability to analyze every cytosine should aid the analyses of correlations between gene expression and DNA methylation (1).

Hydrazine reacts poorly with 5-methylcytosine relative to cytosine and thymine residues (7, 8). Thus, methylation levels of individual cytosine bases in DNA from various tissues can be quantitated from genomic sequence determination autoradiographs. The same DNA replicated in *Escherichia coli* acts as an unmethylated control.

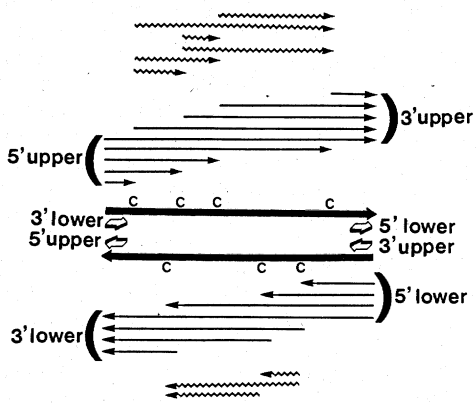

FIG. 1. All the single-stranded DNA reaction products expected in the complete restriction cleavage and partial cytosine-specific cleavage reactions for a restriction fragment. The cytosine residues are indicated by Cs on each of the central genomic DNA strands. Between these strands, four short white arrows indicate the four possible probes for this restriction fragment. The probes are referred to by the type of sequence they will produce. For example, hybridization with the 3′ lower probe produces the image of the 3′ end-labeled sequence of the lower strand. The straight arrows represent reaction products that will contribute to readable sequences only when the probe indicated by adjacent brackets is used. Zigzag lines represent internal reaction products that can only deteriorate the sequence when the probes are made long enough to overlap many of these.

MATERIALS AND METHODS

DNA Samples. To determine the sequence of a specific region of DNA, we use a restriction enzyme that cuts about 100–200 base pairs (bp) away on one side of the region and a greater distance away on the other side. Genomic DNA samples are cut to completion, precipitated by ethanol, and resuspended at 10 μg/μl in 10 mM Tris·HCl, pH 7.5/1 mM EDTA. Five 5-μl aliquots of the DNA are treated with standard G, A+G, T+C, T, and C chemistries (9, 10). After the final lyophilizations, the samples are resuspended at 10

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Abbreviations: C_μ, IgM heavy chain constant region gene; bp, base pair(s).

$\mu g/\mu l$ in 94% formamide/0.05% xylene cyanol/0.05% bromophenol blue/10 mM Na_2EDTA, pH 7.2. With a Hamilton syringe (1701SNWG, 31-gauge, 5-cm, point 3 needle), 2.5 μl of each reaction is loaded on a 50 × 50 × 0.076 cm 6% acrylamide/0.15% bisacrylamide/7 M urea/50 mM Tris borate/EDTA, pH 8.3/0.1% ammonium persulfate/0.1% *N,N,N',N'*-tetramethylethylenediamine gel in 5-mm-wide slots formed by a series of polyacrylamide or acetal plastic dividers 0.5–1 mm wide.

Nylon Membranes. Many filters retain small DNA fragments and resist damage better than does nitrocellulose. DBM (11), DPT (12), Millipore poly(vinylidene fluoride), Bio-Rad Zetaprobe, New England Nuclear GeneScreen plus, AMF Cuno Zetabind, Pall RU, and Pall Biodyne A have been used to produce sequence patterns with loadings that range from nanogram to 20-fg amounts of hybridizable DNA per band. Certain nylon membranes allow us to detect 3 fg of hybridizable DNA per band: among two lots of Pall NR (32 × 50 cm from Chisholm (Cranston, RI) and three lots of GeneScreen (New England Nuclear), we have noticed no significant variation in the signal-to-noise ratio for single-copy sequences.

Electrophoretic Transfer. The following protocol deviates from previous protocols (13–21). We built a 38 × 46 × 20 cm transfer device from Plexiglas egg-crate louver panels (from AIN Plastics (Mt. Vernon, NY), Scotch-Brite pads (96 type-industrial; 3M, Inc., Minneapolis, MN), and 32-gauge platinum wire. Replicas of this device can be obtained from Charles Barbagallo (Harvard Biological Laboratories, Cambridge, MA).

The gel and nylon membrane are kept thoroughly wet with 50 mM Tris borate/EDTA, pH 8.3. The gel can be transferred immediately after completion of the electrophoretic separation of the DNA fragments (removal of urea from the gel, DNA depurination, and cleavage of bisacrylamide cross-linkers are not necessary). The gel, if 0.76 mm thick, is lowered directly from the glass plate onto one Scotch-Brite surface of the transfer device. Thinner gels are lifted with dry Whatman 540 paper and placed immediately on the Scotch-Brite surface. The nylon membrane is placed on the gel without trapping pockets of air or buffer at the interface. Keeping the gel plane horizontal during transfer prevents sagging of the large thin gels and membranes and allows application of pressure from a 2-kg mass to the central region of the transfer assembly to aid tight contact between the gel and nylon surfaces. The power for electrophoretic transfer is supplied by line current with a bridge rectifier, which delivers 108 V at 120 pulses per sec direct current output up to 4 A (available from J. Skare, 665 North St., Tewksbury, MA 01876). The rate of transfer depends on DNA size, but in 30 min with a 108-V potential over the 10 cm between electrodes in 50 mM Tris borate/EDTA, greater than 90% of the DNA fragments, 26 through 516 nucleotides in length, transfer from a 1.5-mm thick 6% polyacrylamide gel.

UV Irradiation. The lower left corner of the wet membrane is clipped to designate that the DNA-coated side is facing up. This side is placed on taut Saran Wrap and irradiated through the Saran Wrap at a distance of 35 cm from one or more germicidal UV bulbs. The UV flux can be measured with a Blak-Ray 260-nm UV meter (from American Scientific Products) or can be determined empirically by using genomic sequence analysis transfers. The optimal UV dose for cross-linking DNA to the filter is 1.6 kJ/m^2, which on our device using six bulbs is 1200 $\mu W/cm^2$ for 2 min. DNA images on completely dry membranes have a 90% lower optimal UV dose.

We dried [α-^{32}P]-labeled NTPs onto nylon membranes and UV-irradiated these at 0.16 kJ/m^2. Nucleotide binding was stabilized 130-fold for TTP and 30-, 20-, and 10-fold for dGTP, dCTP, and dATP, respectively. These bonds were stable for over 14 hr at 65°C at pH 2 through 11. Primary amino groups (which are present on nylon) are highly reactive with 254-nm light-activated thymines (22).

DNA Probe Synthesis. A gel-purified DNA fragment, homologous to sequences to one side of the restriction site selected for the total genomic digests, is subcloned into *Sma* I-cut mp8 vector (23). Large quantities (>500 μg) of single-stranded phage DNA are prepared from individual white plaques, avoiding residual traces of bacterial DNA and polyethylene glycol. The single-stranded DNA is resuspended at 2 mg/ml in 10 mM Tris·HCl/1 mM Na_2EDTA, pH 7.5, and 30 μl of this DNA, 7 μl of synthetic 17-nucleotide-long sequence assay primer from Collaborative Research (Waltham, MA) at 10 ng/μl, and 2 μl of 100 mM $MgCl_2$ are incubated at 50°C for 40 min. Then, 1 μl of crystalline grade bovine serum albumin at 10 mg/ml, 1 μl of 3 mM dCTP/3 mM dGTP/3 mM TTP, 0.1 μl of 200 mM dithiothreitol, 50 μl of [α-^{32}P]dATP at 10 mCi/ml (>5000 Ci/mmol; 1 Ci = 37 GBq), and 3 μl of DNA polymerase I large fragment at 5 units/μl from New England BioLabs or Bethesda Research Laboratories are mixed and incubated at 25°C for 40 min. After addition of 200 μl of 94% formamide/0.05% xylene cyanol/0.05% bromophenol blue/10 mM Na_2EDTA, pH 7.2, the reaction is heated in a boiling water bath, 10 μl of 1 M NaOH is added, and the entire sample is loaded directly (without cooling) onto a 15 × 0.6 × 0.15 cm slot on a 20 × 10 × 0.15 cm 6% acrylamide/0.15% bisacrylamide/7 M urea/50 mM Tris borate/EDTA, pH 8.3/0.1% ammonium persulfate/0.1% *N,N,N',N'*-tetramethylethylenediamine gel that has been preheated to a 50°C surface temperature by preelectrophoresis at 250 V/10 cm and 90 mA. When the bromophenol blue dye reaches 1.4 cm from the origin (about 10 min), the xylene cyanol dye-stained region of the gel is excised, ground with a thick glass rod, and eluted in 10 ml of hybridization buffer (see below) for 45 min at 50°C. Small scale reactions should show greater than 90% of the label incorporated into fragments 90–130 nucleotides in length. If most of the extension reaction products are longer than 150 bp, the primer and template DNA concentrations should be increased and the reaction retested.

RNA Probe Synthesis. The SP6 RNA polymerase transcription is done as described by Zinn *et al.* (24) and modified by K. Zinn. The SP63 vector used contains an SP6 promoter very near a *Hin*dIII cloning site (P. Kreig and D. Melton, personal communication). The following are mixed in order at 24°C: 8 μl of mix [90 mM Tris·HCl, pH 7.5/14 mM $MgCl_2$/5 mM spermidine·HCl/1.3 mM ATP/1.3 mM CTP/1.3 mM GTP/22 mM dithiothreitol/0.6 units of Promega Biotec (Madison, WI) RNasin per ml], 10 μl of [α-^{32}P]rUTP at 50 mCi/ml (3000 Ci/mmol; partially lyophilized from 50 μl), 2 μl of restricted DNA template at 1 mg/ml in H_2O, and 0.7 μl of SP6 RNA polymerase at 7 units/μl. The reaction is incubated at 37°C for 1 hr, then 8 μl of mix, 10 μl of H_2O, 2 μl of DNA template, and 0.5 μl of enzyme are added, and the reaction mixture is incubated an additional hour. Fifty-percent incorporation of the label should be achieved. The reaction mixture is extracted with phenol, and the extracted DNA is precipitated and rinsed with ethanol, dried, and resuspended in 50 μl of 10 mM Tris·HCl/1 mM EDTA before dilution in 5 ml of hybridization buffer. The enzyme is available from New England Nuclear and Promega Biotec.

Hybridization. Gel elution and hybridization buffer is 1% crystalline grade bovine serum albumin/1 mM EDTA/0.5 M $NaHPO_4$, pH 7.2/7% $NaDodSO_4$ [1 M $NaHPO_4$ (pH 7.2) stock is composed of 134 g of $Na_2HPO_4 \cdot 7H_2O$ and 4 ml of 85% H_3PO_4 per liter]. Either Pierce 28365 or Bio-Rad 161-0302 $NaDodSO_4$ is adequate. Carrier DNA, RNA, pyrophosphate, Dextran sulfate, Ficoll, and polyvinylpyrrolidone are not required in these hybridizations. Bovine serum albumin has only a 2-fold effect on background. The UV-irradiated

nylon membrane is placed in a polyethylene/polyester-laminated bag (18 × 15 inch Scotchpak 229 from Spec-Fab (Riverton, NJ) and rinsed with water. Excess water is removed before addition of 30 ml of hybridization buffer. The bag is heat-sealed (model 254-B 1/4 × 24 inch Teflon jaws from Clamco, Cleveland, OH) for 4 sec at 250°F and placed at 65°C for 5 min. The gel particles are removed from the eluted probe by centrifugation at 3000 × *g* for 3 min and by rapid filtration of the supernatant through a 0.22-μm Millex-GV filter. The 30 ml of hybridization buffer in the bag is replaced by the probe. The bag is resealed, incubated at 65°C for 8–24 hr in a water bath, reopened, and submerged in 1 liter of 0.5% fraction V-grade bovine serum albumin/1 mM Na_2EDTA/40 mM $NaHPO_4$, pH 7.2/5% $NaDodSO_4$. After 5 min with agitation, the membrane is transferred to another such wash, followed by eight washes (1 liter each) in 1 mM Na_2EDTA/40 mM $NaHPO_4$, pH 7.2/1% $NaDodSO_4$ for 5 min each. For the RNA probes, three additional washes in 100 mM $NaHPO_4$ (pH 7.2) to remove $NaDodSO_4$ and treatment with 30 ml of RNase A (10 μg/ml) in 0.3 M NaCl/10 mM Tris·HCl/1 mM Na_2EDTA, pH 7.5, at 37°C for 15 min were done.

The stated volumes are intended for one nylon sheet measuring 30 × 40 cm. When the total membrane surface area is different, the probe synthesis, hybridization, and wash volumes are adjusted proportionally. The wash solutions are kept at 65°C prior to use, but the wash agitations are done at room temperature for convenience. The last wash is brought to 65°C by sealing the membrane and wash solution in a bag and submerging this in a water bath for 20 min. The total Na^+ concentration in this wash is 76 mM. The dry membrane is autoradiographed on preflashed XAR-5 film at −80°C with an intensifying screen (25) for 2 or more days. The probe can be eluted by washing in 500 ml of 2 mM Tris/EDTA, pH 8.2/0.1% $NaDodSO_4$ for 15 min at 65°C, and then the membrane can be reprobed.

RESULTS AND DISCUSSION

An Example. We have studied the methylation of cytosines in a region from the mouse IgM heavy chain constant region gene C_μ in five cell types. Fig. 2 shows the probes for the ends of a 511-bp *Mbo* I fragment that covers the third intron of C_μ. Fig. 3 shows the genomic sequencing patterns. DNA in lanes of Fig. 3 *Left* are hybridized to the 110-nucleotide long 5′ lower probe (Fig. 2); then the membrane was stripped and rehybridized (Fig. 3 *Right*) with the 256-nucleotide-long RNA transcript corresponding to the 3′ lower probe (Fig. 2). The lanes in Fig. 3 marked l (liver) display the base-specific (guanine, purine, pyrimidine, thymine, and cytosine) reactions on 25 μg of liver DNA. The subsequent lanes displayed are cytosine-specific reactions on DNA from thymus (t), spleen cells sorted for surface IgM, positive and negative fractions (m and n), and RAW 8.1 lymphosarcoma cells (r).

The genomic clone (pIgMC1) used as the unmethylated control (Fig. 3, lanes p) was derived from the BALB/c cell line 18-48 (29). We analyzed tissues from 3-month-old female mice of the same inbred BALB/c strain. Three groups independently have reported sequence data for the C_μ region of BALB/c mice (30–32). Although these three sequences differ at some positions, they agree at the positions of the C-G dinucleotides that we have analyzed. Interlane variations in cytosine band intensities are apparent at the C-G dinucleotides at positions 192, 325, and 401 (see arrowheads in Figs. 2 and 3). The cytosine at 192 is normally reactive (unmethylated) in liver and in the IgM-positive spleen cells and is hyporeactive (methylated) in the thymus, a lymphosarcoma, and the surface IgM-negative spleen cells. The cytosine at 401, part of a *Hpa* II site, appears methylated in all cell types, but

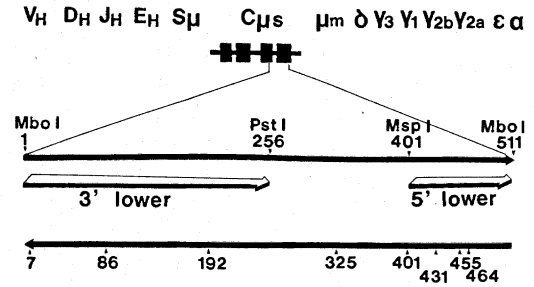

FIG. 2. Mouse C_μ-specific probes. The four exons of the C_μ DNA (solid black blocks) are placed in the context of the other gene segments required for heavy chain gene expression and recognition diversity [not to scale (V, variable; D, diversity; J, joining; E, enhancer; S, switch; $C_\mu s$–α, constant domains); see ref. 26 for a review]. The 511-bp *Mbo* I fragment at the 3′ end of C_μ is shown enlarged as two long black arrows. Between these strands the extents and polarities of the two probes are indicated by white arrows. The numerals beneath the lower strand indicate the positions of C-G dinucleotides relative to the 3′ end of this strand. Three of these are present in the sequences in Fig. 3: 192, 325, and 401. The plasmid pIgMC1 was constructed from pBR322 and a 5.0-kbp *Bam*HI–*Eco*RI fragment from genomic DNA of Abelson virus-transformed 18-48 BALB/c cell line (provided by A. Perlmutter). The 5′ lower probe was synthesized as described in *Materials and Methods* on a single stranded template from an mp8 recombinant phage, pIgM511Mbo-5′lower, containing a 110-bp *Msp* I–*Sau*3A fragment from pIgMC1 cloned into mp8 with the *Hpa* II site 33 nucleotides from the synthetic primer binding site. The 3′ lower RNA probe was synthesized from the plasmid, pIgM511Mbo3′lower, which was constructed from *Hin*dIII-cut pSP63 (provided by P. Krieg and D. Melton) and the 511-bp *Sau*3AI fragment of pIgMC1. All 3′ ends were filled in before ligation. The recombinant plasmid cut with *Pst* I acted as template for transcription.

in the IgM-positive cells (where this site is less clear), a small degree of unmethylation is not ruled out. The cytosine at 325 produces no bands (less that 10% reactivity by densitometry compared to normal intensities in the control plasmid cytosine-specific reaction—lane p) in all mouse cell types analyzed, consistent with high levels of methylation. Thus, within a space of 133 nucleotides, we find two C-G dinucleotides (positions 192 and 325) with different tissue-specific DNA methylation. Previous studies on C_μ gene DNA methylation (33–35) were limited to *Hpa* II cleavage sites.

Unambiguous sequence can be read for the liver DNA at positions 368–399 when hybridized with the 110 nucleotide long pIgM511Mbo5′ lower probe (Fig. 3 *Left*). When the DNA was rehybridized with the 256-nucleotide-long RNA probe (Fig. 3 *Right*), the lane labeled G became less readable, even though the C lanes were clear in both hybridizations. This probably represents hybridization to internal fragments with both ends produced by chemical cleavages (zigzag lines in Fig. 1), which affected one G lane because of the combined effects of overreaction (about one hit per 80 nucleotides rather than the recommended one per 500) and a long probe.

Other Applications. The genomic sequencing techniques will allow analysis of enzymatic and chemical inhibition enhancement patterns ("footprinting") of the chromatin over single genes in whole cells or nuclei at single nucleotide resolution. By determining the sequence of genomic DNA with the single-base-specific chemical reactions, genetic polymorphisms and somatic mutations should be detectable even in heterozygotes. The UV irradiation of nucleic acids on pure nylon membranes and the use of high $NaDodSO_4$ washes should be helpful in a variety of filter hybridization studies that require high sensitivity and reprobing. Gel fractionation of genomic restriction digests will permit sequence determination of repetitive elements or DNA from organisms

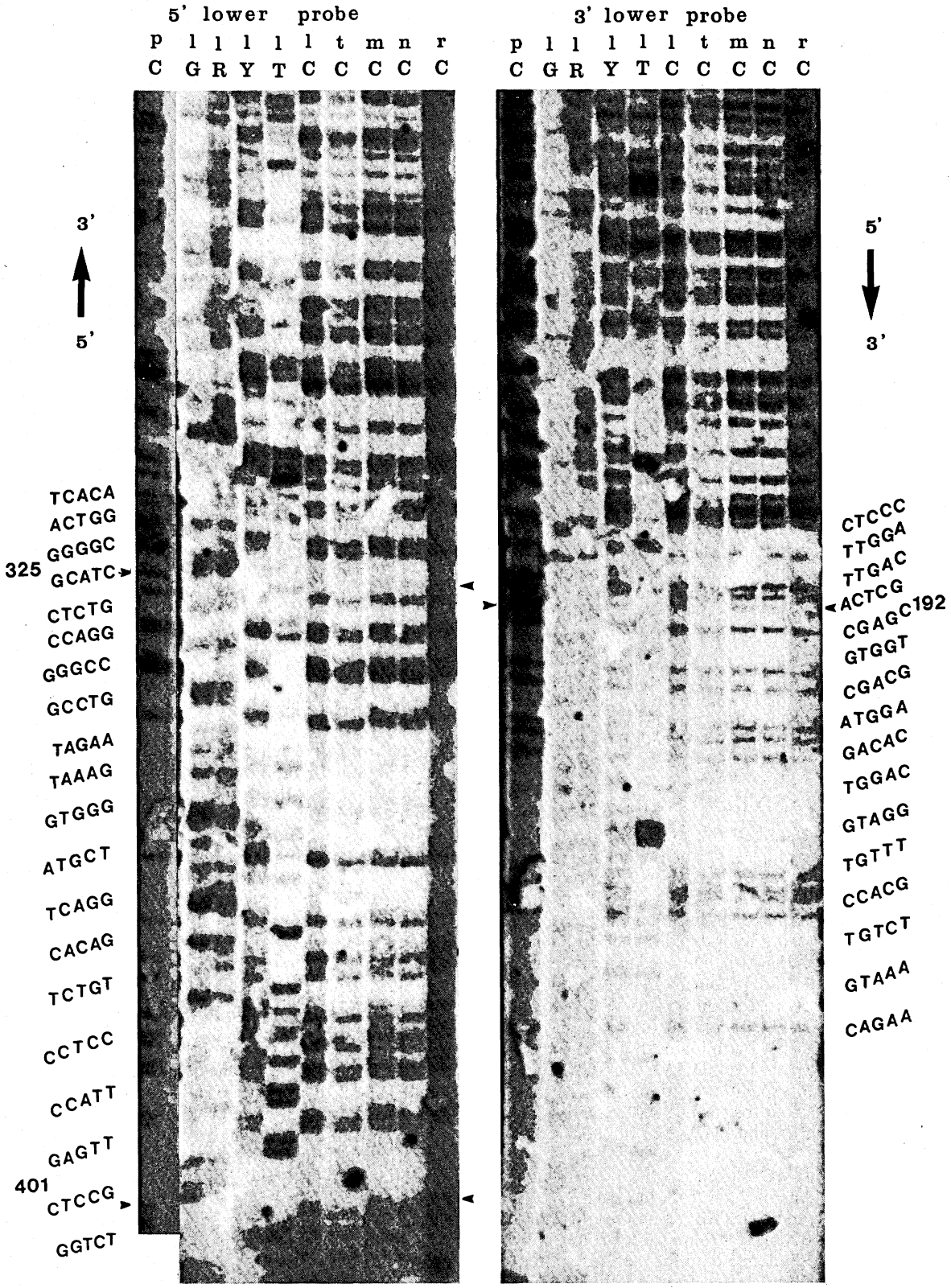

FIG. 3. Genomic sequences of mouse C_μ genes. The symbols p, l, t, m, n, and r represent DNA samples from plasmid pIgMC1, liver cells, thymus cells, spleen cells sorted for presence of surface IgM (27), spleen cells sorted for lack of surface IgM, and BALB/c lymphosarcoma RAW 8.1 TIB 50 cells (28), respectively, which were cut to completion with *Mbo* I or *Sau*3A I. The symbols C, G, R (A+G), Y (C+T), and T ($KMnO_4$) indicate the standard chemical sequence determination reactions used (9, 10). Twenty-five micrograms of genomic DNA or 200 pg of plasmid DNA were loaded per lane. Arrows indicate the positions of C-G dinucleotides; the sequences (from 27–29) are aligned along the extreme right and left. (*Left*) DNA in these 10 lanes was probed with the 5′ lower DNA probe (Fig. 2). The probe concentration during hybridization was 6 μCi/ml = 10 ng/ml = 1.3×10^7 dpm/ml. Exposure time was 21 days. (*Right*) The membrane was stripped of the first probe and rehybridized with the 3′ lower RNA probe. The probe concentration during hybridization was 24 μCi/ml. The exposure time was 10 days.

with more than 3×10^9 bp per haploid genome equivalent. In conjunction with enzyme-linked probes (36), it may be possible to determine the sequence of DNA without using radioactive compounds.

Genomic DNA sequence determination has been successfully applied to other systems: rat insulin II gene DNA methylation (H. Nick and R. Cate, personal communication); DNA methylation maintenance of *in vitro* methylated human fetal globin gene transfected into mouse L cells (ref. 6; unpublished data); *lac* operator DNA accessibility to dimethyl sulfate in intact *E. coli* cells (H. Nick, personal communication); and accessibility of a human β-interferon gene–bovine papillomavirus construct to dimethyl sulfate in mouse C127 cells (K. Zinn and T. Maniatis, personal communication).

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