Nucleic Acids Research

Detection and mapping of homologous, repeated and amplified DNA sequences by DNA renaturation in agarose gels

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ABSTRACT
        A new molecular hybridization approach to the analysis of complex
genomes has been developed. Tracer and driver DNAs were digested with the
same restriction enzyme(s), and tracer DNA was labeled with ^{32}P using T4 DNA
polymerase. Tracer DNA was mixed with an excess amount of driver, and the
mixture was electrophoresed in an agarose gel. Following electrophoresis, DNA
was alkali-denatured in situ and allowed to reanneal in the gel, so that
tracer DNA fragments could hybridize to the driver only when homologous driver
DNA sequences were present at the same place in the gel, i.e. within a
restriction fragment of the same size. After reannealing, unhybridized
single-stranded DNA was digested in situ with S1 nuclease. The hybridized
tracer DNA was detected by autoradiography. The general applicability of this
technique was demonstrated in the following experiments. The common EcoRI
restriction fragments were identified in the genomes of E. coli and four other
species of bacteria. Two of these fragments are conserved in all
Enterobacteriaceae. In other experiments, repeated EcoRI fragments of
eukaryotic DNA were visualized as bands of various intensity after
reassociation of a total genomic restriction digest in the gel. The situation
of gene amplification was modeled by the addition of varying amounts of λ
phage DNA to eukaryotic DNA prior to restriction enzyme digestion.
Restriction fragments of λ DNA were detectable at a ratio of 15 copies per
chicken genome and 30 copies per human genome. This approach was used to
detect amplified DNA fragments in methotrexate (MTX)-resistant mouse cells and
to identify commonly amplified fragments in two independently derived
MTX-resistant lines.

INTRODUCTION

        Electrophoretic separation of DNA fragments produced by restriction

endonuclease digestion is an important tool in studies of DNA structure and

function. Following immobilization of DNA fragments by transfer onto

nitrocellulose filters (1) or in dried agarose gels (2) the restriction

fragments can be hybridized to the exogenously added DNA or RNA probe. This

technique, however, requires that the probe sequences be present only in a

limited number of fractionated DNA fragments so that an interpretable

hybridization pattern can be obtained. This problem arises, in particular,

when a genomic clone of eukaryotic DNA is used as a probe for hybridization

with a restriction digest of total genomic DNA. In this case, the possible presence in the genomic clone of short interspersed repetitive sequences that frequently occur in the DNA of higher eukaryotes (2) results in a smeared pattern due to cross-hybridization of the repetitive sequences.

Recent studies demonstrated that amplification of specific DNA sequences is a common mechanism for adaptation of eukaryotic cells to a variety of selective conditions. Gene amplification was also found to occur in some developmental processes and was suggested as a possible mechanism of carcinogenesis and tumor progression (4-7). A general method for detection and characterization of amplified DNA sequences is needed for the analysis of those systems where the nature of amplified genes is unknown and no cloned probes are available. The existing approaches to this problem include purification of chromosomal structures that are known to contain amplified DNA, i.e, double minute chromosomes (8) or unusually large chromosomes with homogeneously staining regions (9) and cloning amplified DNA sequences by differential screening with genomic probes (10). None of these techniques permits rapid detection of amplified genes in cellular DNA preparations or comparison of amplified sequences between different DNA preparations prior to cloning. Following electrophoretic separation of a total restriction digest of eukaryotic DNA and ethidium bromide staining of DNA, those restriction fragments that are repeated at least several hundred times per vertebrate genome, can be detected as distinct bands against the background smear. A more sensitive assay is required for identification of selectively amplified DNA fragments, the copy number of which is increased between two or three and two hundred times in most studied cases of gene amplification.

The present article describes a new method of DNA hybridization that combines the advantages of the analysis of separated restriction fragments with the ability to use complex eukaryotic and prokaryotic genomes both as a tracer and as a driver. Restriction fragments of radioactively labeled tracer and unlabeled driver DNAs are co-electrophoresed in an agarose gel and hybridized in situ after electrophoresis so that hybridization can occur only between DNA fragments of the same size. Hybridization of different bacterial DNAs by this procedure permits identification of common restriction fragments in these genomes. When reassociation of a restriction digest of total eukaryotic DNA is analyzed by this technique, repeated fragments present in sufficient concentration to effectively reanneal in the gel can be detected. In model experiments, precisely repeated DNA sequences could be visualized at a frequency as low as 15 copies per chicken genome or 30 copies per human

genome. Comparison of the repeated fragments in the DNA of mouse 3T3 cells
and its methotrexate (MTX)-resistant derivatives reveals a set of additional
bands corresponding to amplified DNA fragments in drug-resistant cells.
Hybridization of DNA from two independently derived MTX-resistant cell lines
reveals a set of commonly amplified fragments in these two lines. These
results suggest that this technique can be used as a general assay for gene
amplification.

MATERIALS AND METHODS

Restriction endonucleases and the large fragment of DNA polymerase I
were obtained from New England Biolabs. T4 DNA polymerase was purchased from
Bethesda Research Laboratories and S1 nuclease was from Sigma. $[\alpha^{-32}P]$ dCTP
(400-3,000 Ci/mM in 50% ethanol) was obtained from Amersham. Formamide,
obtained from Kodak (P565), was deionized with Bio-Rad RG501-X8 ion-exchange
resin.

DNA from recombinant plasmids $pCG_\rho 1$ and $pCG_\epsilon 1$, containing chicken
embryonic β-globin cDNA sequences inserted into pBR322 (11,12) was prepared as
described (13). DNA from a recombinant phage λw143, containing a portion of
the chicken α-globin cluster in λ Charon 4A, was a gift from L. S. Haigh. λ
phage DNA was obtained from New England Biolabs. Chicken DNA, extracted from
the whole carcass of 10-day-old embryos was a gift from S. Hellewell, and DNA
from an Epstein-Barr virus transformed human lymphocyte line Gus 5 was a gift
from D. Housman. DNA from mouse NIH 3T3 cells and from the MTX-resistant line
R500 (27) was extracted by the procedure of Blin and Stafford (10). Nuclear
DNA from the MTX-resistant line R.3 was a gift of R.M. Snapka. Bacterial DNA
preparations were gifts of S. J. Elledge, J. H. Thomas, N. Neff and R.
Bourret. DNA concentration was determined by diphenylamine titration (15).
The number of the DHFR gene copies in DNA from MTX-resistant cells was
determined by dot blot hybridization (R.M. Snapka and A. Varshavsky,
unpublished results).

Single-stranded end-labeled fragments of the plasmids $pCG_\rho 1$ and $pCG_\epsilon 1$
were prepared by digesting these plasmids with HincII and MspI, followed by 3'
end-labeling using the large fragment of DNA polymerase I and $[\alpha^{-32}P]$ dTTP
(11). The labeled fragments were purified from agarose gels as described (12)
and strand-separation was performed according to the procedure of Maxam and
Gilbert (16).

DNA labeling and gel electrophoresis. Restriction enzyme digestion was done
under conditions recommended by the supplier. To ensure complete digestion, 5

units of enzyme per 1 μg of DNA were used. Tracer DNA was labeled by replacement synthesis with T4 DNA polymerase and [α-^{32}P] dCTP (17). In the exonuclease reaction, 0.2-2 μg of DNA were incubated with 2 units of T4 DNA polymerase in 10 μl of reaction buffer (33 mM Tris-acetate pH 7.9/ 65 mM Na acetate/ 10 mM Mg acetate/ 100 μg/ml bovine serum albumin/ 0.5 mM dithio-treitol) at 37°C. The time of the exonuclease reaction varied from 2 to 30 minutes. For the resynthesis reaction, the mixture was transferred into a tube containing 100-200 μCi of dry [α-^{32}P] dCTP, and dATP, dGTP and dTTP were added each to 80 μM in a final volume of 25 μl of the reaction buffer. After incubation at 37°C for 30 min, unlabeled dCTP was added to 80 μM, and the reaction was continued for 20 minutes in order to ensure complete regeneration of the duplexes. The reaction was stopped by the addition of 4 volumes of 2.5M ammonium acetate and 10 μg tRNA, and DNA was precipitated with 3 volumes of ethanol. The pellet was redissolved in 150 μl of 0.3 M Na acetate, and DNA was ethanol precipitated for the second time. The final pellet was dissolved in a convenient volume of 10 mM Tris-HCl, pH 7.5/ 1 mM EDTA.

The amount of tracer DNA used for electrophoresis varied from 500 dpm in the case of phage DNA digests to 5-15 x 10^6 dpm for the digests of eukaryotic DNA. Tracer DNA was mixed with varying amounts of driver DNA, as described in Results, and the mixtures were loaded onto a horizontal 21.9 x 15.3 x 0.4 cm slab gel of 1% agarose (Bethesda Research Laboratories, gel electrophoresis grade) in Tris-acetate buffer (22.5 mM Tris-acetate, pH 8.3/ 10 mM Na acetate/ 1 mM EDTA). The sample wells were either 6.4 mm wide and 1 mm thick or 8.2 mm wide and 1.2 mm thick. The sample volume was 12 μl in the first case and 18μl in the second case. Electrophoresis was performed in Tris-acetate buffer containing 0.5 mg/l ethidium bromide at 50 v. Following electrophoresis, gels were photographed using a 366 nm wavelength ultraviolet illuminator.

In gel hybridization. The gel slab was cut to a 18.6 x 12.3 cm size and carefully transferred into a 19.0 x 12.7 x 4.3 cm flat bottom polystyrene box. The close correspondence between the sizes of the gel slab and the box was essential in order to prevent gel breakage in the course of shaking. All of the following procedures, unless specified otherwise, were done at 37°C with constant shaking on a rotary table at a speed of 40-100 rpm. The solutions were warmed to 37°C prior to use.

DNA was denatured by soaking the gel in 150 ml of denaturing buffer containing 0.5 M NaOH/ 0.6 M NaCl/ 0.004% thymol blue. After 30 min. incubation, the buffer was removed by aspiration, the same volume of fresh denaturing buffer was added, and incubation was continued for another 30 min.

The gel was neutralized by washing with 150 ml volumes of the hybridization buffer, containing 50% formamide/ 3 x SSPE (1 x SSPE: 10 mM Na phosphate, pH 7.0/ 0.18 M NaCl/ 1 mM EDTA), four or five times for 15-20 minutes each time. Neutralization of the gel was initially indicated by the appearance of the yellow color of thymol blue in the gel, after which the pH of the washing buffer was monitored with pH indicator sticks. Following neutralization, the gel was incubated in 100 ml of the hybridization buffer for a period of 2 to 20 hours, during which time DNA renaturation occured. In some experiments, the composition of the hybridization buffer was 5 x SSPE or 10 x SSPE/ 50% formamide, and hybridization was performed at 45°C.

After hybridization, the gel was washed with 150 ml of the S1 nuclease digestion buffer (50 mM Na acetate, pH 4.6/ 0.2 M NaCl/ 1 mM $ZnSO_4$) four times for 20 minutes each time. S1 nuclease digestion was done in 100 ml of the same buffer containing 50-100 units/ml S1 nuclease for 2 hours.

Following S1 nuclease treatment, the digestion products were eluted from the gel by washing with 100-150 ml volumes of 3 x SSPE, pH 7.0/ 0.1% $NaDodSO_4$ for a total of 2 hours with three changes of buffer. After elution, the gel was dried in a gel slab dryer (Bio-Rad) and autoradiographed using Kodak XAR-5 film with an intensifying screen. In some experiments, immediately after S1 nuclease digestion the gel was placed in the denaturing buffer, and the above hybridization procedure and S1 treatment were repeated.

RESULTS

Two factors are crucial for successful renaturation of DNA in agarose gels: tracer DNA fragments should be undegraded so that their electrophoretic mobility would be the same as for the driver DNA fragments, and the unhybridized single-stranded DNA should be competely removed from the gel by S1 nuclease digestion. The first condition is fulfilled by using the replacement synthesis procedure (18) for DNA labeling. This technique, which includes exonucleolytic degradation of DNA using the 3'→5' exonuclease activity of T4 DNA polymerase followed by efficient resynthesis of degraded ends, allows one to obtain intact DNA fragments of high specific activity. An additional advantage of this procedure is that the distribution of label in the fragments can be controlled by varying the extent of the exonuclease reaction. As discussed below, limiting the label to the terminal portions of restriction fragments may be essential in some experiments.

In order to determine the optimal conditions for in situ digestion of DNA by the single-strand specific nuclease S1, both double-stranded and
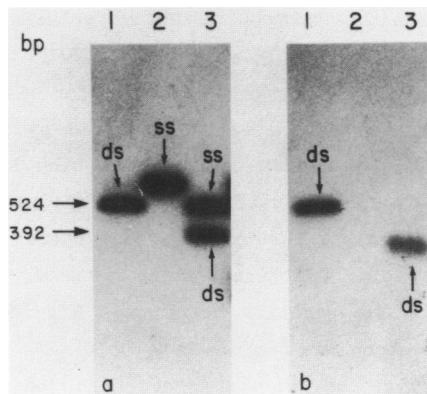
Fig. 1.   In situ digestion of single-stranded DNA with S1 nuclease.   3'
end-labeled 524 bp fragment of the plasmid pCG$_\rho$1 and 392 bp fragment of the
plasmid pCG$_\varepsilon$1 were prepared, and labeled strands were purified as described in
Materials and Methods.
a) Autoradiogram of a 1.5% agarose gel. Lanes: 1 - double-stranded 524 bp
fragment; 2 - single-stranded 524 bp fragment; 3 - mixture of single-stranded
and double-stranded 392 bp fragments. ds: double-stranded. ss: single-
stranded.
b) A duplicate gel washed three times with S1 nuclease buffer and incubated
with 50 units/ml S1 nuclease for 2 hours. Lanes 1-3 are as in (a).

single-stranded end-labeled DNA fragments were prepared from recombinant
plasmids pCG$_\rho$1 and pCG$_\varepsilon$1, as described in Materials and Methods.   Double-
stranded (ds) and single-stranded (ss) fragments were co-electrophoresed in a
1.5% agarose gel (Fig. 1a). Duplicate gels were washed with the S1 nuclease
digestion buffer and incubated with varying amounts of S1 nuclease. Fig. 1b
shows that incubation with 50 units/ml S1 nuclease for 2 hours, followed by
elution of the digested material from the gel, results in complete removal of
single-stranded DNA. These conditions were used for degradation of denatured
DNA following in gel renaturation.

The conditions for DNA renaturation in agarose gels were assayed in the
experiments of the type shown in Fig. 2.   In this experiment, DNA of a
recombinant phage λw143 was digested with restriction enzymes EcoRI and
HindIII and labeled with ^{32}P by replacement synthesis.   Equal amounts of
labeled DNA (tracer) were mixed with increasing amounts of the same unlabeled
DNA (driver) and electrophoresed in a 1% agarose gel. DNA was hybridized in
the gel as described in Materials and Methods. As can be seen in Fig. 2,
protection of tracer DNA from S1 nuclease digestion is dependent upon the
amount of driver DNA present in the gel. Addition of 10 μg of EcoRI-digested
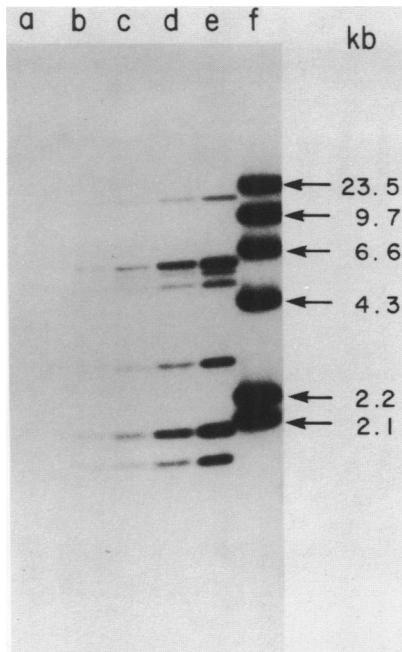
Fig. 2. In gel renaturation of phage DNA fragments. λw143 DNA was digested with EcoRI and HindIII and labeled to the specific activity 1.05 x 10^7 dpm/μg DNA. 47.2 pg of labeled tracer DNA (corresponding to 2 pg of the 2 kb fragment) were mixed with increasing amounts of the same unlabeled DNA used as a driver, electrophoresed in 1% agarose and reassociated in the gel as described in Materials and Methods. The total amount of DNA in the 2 kb fragment was: 2 pg (lane a), 10 pg (lane b), 50 pg (lane c), 200 pg (lane d) and 1 ng (lane e). HindIII fragments of λ DNA (2,500 dpm in 0.6 μg) were used as size standards (lane f). Autoradiography was done for 36 hours.

salmon sperm DNA to the mixture prior to electrophoresis did not result in additional protection of tracer DNA (data not shown). This result indicates that tracer DNA was indeed retained in the gel due to hybridization with homologous driver DNA rather than non-specifically protected from S1 nuclease by the presence of excess unlabeled DNA in the gel. The minimum amount of DNA detectable under the conditions of the experiment shown in Fig. 2 (hybridization in 3 X SSPE/ 50% formamide at 37°C) was between 5 and 10 pg per band (as determined for restriction fragments of 2 kb size). This level of sensitivity was achieved after 2 hours of hybridization following neutralization of the gel. Hybridization for longer periods of time (up to 20 hours) resulted in only a slight increase in the intensity of tracer DNA bands probably as a result of partial elution of DNA from the gel in the course of
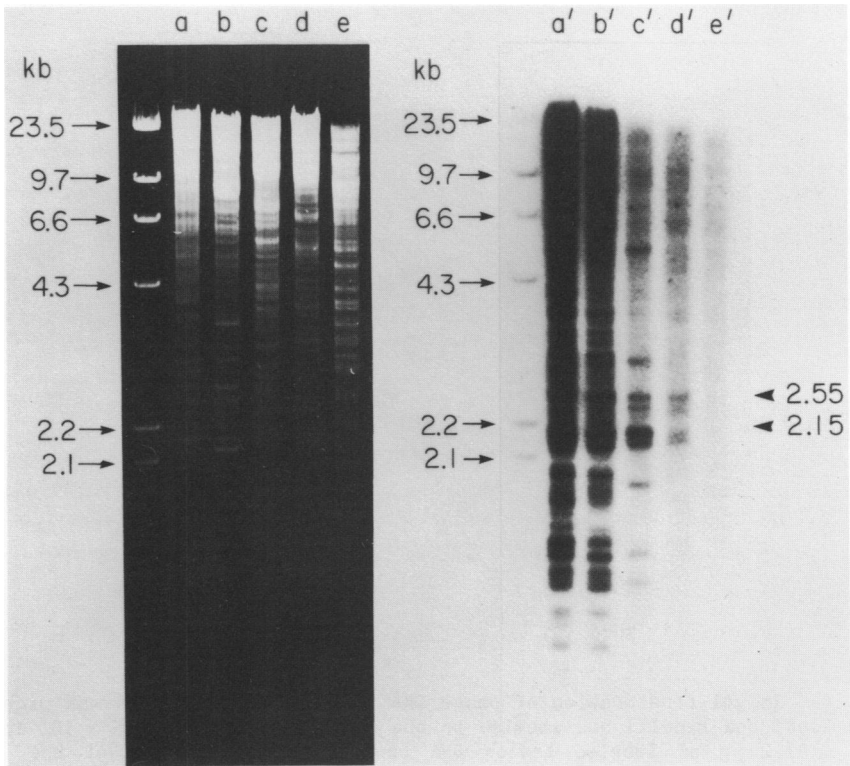
Fig. 3. Identification of common EcoRI restriction fragments in bacterial genomes. E. coli DNA was digested with EcoRI and labeled to a specific activity 7.6×10^7 dpm/μg under the conditions when an average of 750 nucleotides were excised from each 3' end in the exonuclease reaction. 4 ng (3×10^5 dpm) of E. coli tracer DNA were mixed with 2.5 μg of EcoRI digests of the following driver DNAs: E. coli (lane a/a'), Shigella flexneri (lane b/b'), Salmonella typhimurium (lane c/c'), Proteus mirabilis (lane d/d') and Staphylococcus aureus (lane e/e'). Lanes a-e: ethidium bromide staining pattern of the gel prior to hybridization. Lanes a'-e': autoradiogram of the dried gel after in gel hybridization. Hybridization was performed in 3 x SSPE/ 50% formamide for two hours at 37°C. 100 units/ml S1 nuclease were used. HindIII fragments of λ DNA were used as size standards. Arrowheads: EcoRI fragments present in the DNA of all four Enterobacteriaceae. Autoradiography was done overnight.

prolonged incubation. Hybridization under the conditions of higher ionic strength (10 x SSPE/ 50% formamide at 45°C) allowed one to detect as little as 2.5 pg DNA per band (data not shown). Variation in the intensity of different bands in Fig. 2 is due primarily to the fact that some fragments were less efficiently labeled with T4 DNA polymerase than the others. Another

consistently observed artifact was a lower sensitivity of the technique for large (>15 kb) DNA fragments, which was probably due to the preferential sensitivity of large fragments to mechanical degradation and radiolysis of tracer DNA.

This technique was used to identify common EcoRI restriction fragments, i.e. homologous fragments of the same size (within the limits of resolution of an agarose gel), between the DNA of E. coli (4×10^3 kb long) and four other species of bacteria. Genomes of these bacteria are characterized by a different degree of overall sequence homology to the genome of E. coli, as determined by solution and filter hybridization (19). DNA of E. coli strain AB 1157, that does not contain any detectable plasmid DNA (S. J. Elledge, personal communication), was digested with EcoRI and used as a tracer. EcoRI digests of the following DNAs were used as drivers: E. coli, Shigella flexneri, Salmonella typhimurium, Proteus mirabilis and Staphylococcus aureus. Shigella DNA shares 86% sequence homology with E. coli, and the number of common EcoRI fragments between these genomes is too high to be counted (Fig. 3b'); the degree of hybridization, however, is clearly below that observed in the lane where E. coli DNA was used as a driver (Fig. 3a'). Salmonella DNA is 38% homologous to E. coli, however, only 10 common EcoRI fragments ranging in size from 5.2 to 0.75 kb could be detected (Fig. 3c'). The DNA of Proteus is only 5% homologous to E. coli, and it contains four EcoRI fragments that hybridize to the same size fragments of E. coli DNA, though the weaker appearance of the corresponding bands suggests that their homology is incomplete (Fig. 3d'). Staphylococcus aureus is the only species of those analyzed that does not belong to the family of Enterobacteriaceae, and its DNA does not reveal any EcoRI fragments common with E. coli DNA (Fig. 3e'). The presence of labeled material at the top of lanes c', d' and e' in Fig. 3 is due to residual reassociation of tracer DNA, and it was significantly decreased when lower amounts of tracer DNA were used (data not shown). Two EcoRI fragments of a 2.55 and 2.15 kb size (indicated with arrows) appear to be shared by the DNAs of all four Enterobacteriaceae. It would be interesting to determine the nature of these highly conserved DNA sequences.

The applicability of the procedure of DNA renaturation in agarose gels to the analysis of amplified and repeated sequences in the DNA of higher eukaryotes was demonstrated in the experiment shown in Fig. 4, where the situation of gene amplification was modeled by the addition of varying amounts of λ phage DNA to chicken genomic DNA. Chicken DNA, as well as mixtures of chicken and λ phage DNAs prepared at the ratios of 15 or 100 λ genomes (49 kb
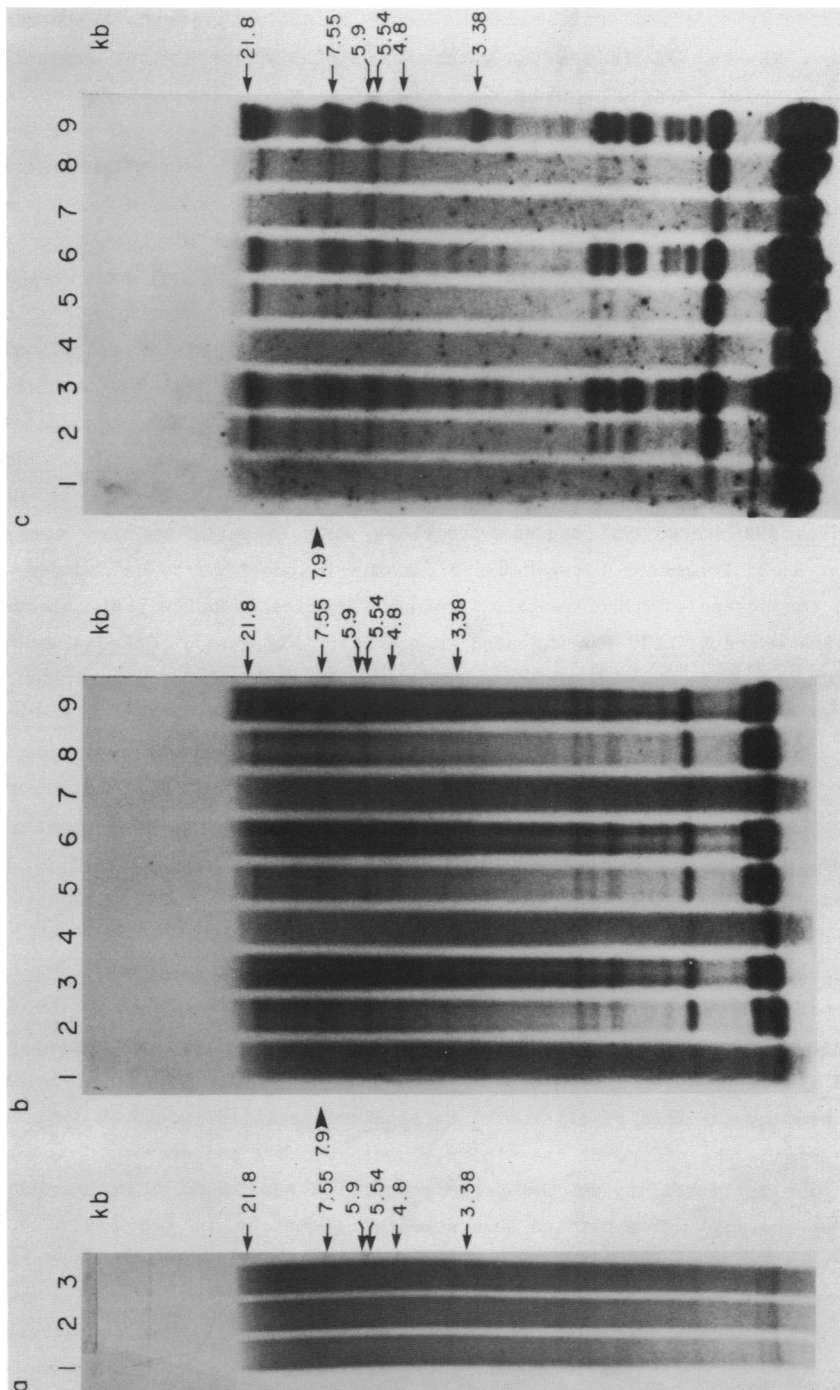
size) per haploid amount of chicken DNA (1.05×10^6 kb), were digested with EcoRI. Tracer DNAs were labeled under the conditions of limited exonuclease digestion so that incorporation of [α-^{32}P] dCTP was confined to a stretch of approximately 100 bp from the ends of restriction fragments. Tracer DNA was mixed with increasing amounts of the same unlabeled DNA used as a driver and hybridized in the gel following electrophoresis in 1% agarose. The reason for limiting the label to the ends of restriction fragments was that short interspersed repetitive sequences present in chicken DNA (20), as well as in other eukaryotic DNAs, were unlikely to acquire the label under these conditions, unless such sequences contained an EcoRI restriction site or were located in the immediate vicinity of such sites. As a result, partial duplexes that can be formed by restriction fragments of the same size containing homologous repetitive sequences along with different unique sequences, would usually have the label only in their single-stranded regions, and therefore would not be detected after S1 nuclease digestion.

An autoradiogram of a dried gel containing labeled EcoRI digests of chicken DNA and of the mixtures of chicken and λ phage DNAs prior to in gel hybridization is shown in Fig. 4a. Several bands corresponding to the highly repeated chicken DNA fragments can be visualized in the lower part of the gel. The EcoRI fragments of λ DNA can also be detected in the mixture of 100 λ DNA

Fig. 4. Detection of repeated EcoRI fragments in chicken DNA and in mixtures of chicken and λ phage DNAs. Chicken DNA and mixtures of chicken and λ DNA at genomic ratios of 1 chicken: 15 λ DNA and 1 chicken: 100 λ DNA were digested with EcoRI and labeled to a specific activity 10^8 dpm/μg under conditions when an average of 100 nucleotides were excised from each 3' end in the exonuclease reaction.
a) Autoradiography of tracer DNAs electrophoresed in a 1% agarose gel. Lane 1: chicken DNA. Lane 2: chicken + λ DNA (ratio 1:15). Lane 3: chicken + λ DNA (ratio 1:100). Position and sizes of EcoRI fragments of λ DNA are indicated.
b) Tracer DNA (10^6 dpm in each lane) was mixed with increasing amounts of the same unlabeled DNA used as a driver, electrophoresed in 1% agarose and hybridized in the gel in 3 x SSPE/ 50% formamide at 37°C for 2 hours after neutralization. 100 units/ml of S1 nuclease were used. Lanes 1-3: chicken DNA. Lanes 4-6: chicken + λ DNA (ratio 1:15). Lanes 7-9: chicken + λ DNA (ratio 1:100). The total amount of DNA in each lane was 10 ng (lanes 1,4,7), 700 ng (lanes 2,5,8) and 7 μg (lanes 3,6,9). Arrowhead: the 7.9 kb band corresponding to the reported EcoRI fragment containing chicken ribosomal DNA (21). Autoradiography was done overnight.
c) Lanes are same as in (b) except that the total amount of DNA in lanes 1, 4, 7 was 90 ng and each lane contained 9×10^6 dpm of tracer DNA. DNA was subjected to in gel renaturation as in (b) except that 50 units/ml S1 nuclease were used. Immediately after S1 nuclease digestion DNA in the gel was again denatured, hybridized for 11 hours after neutralization and digested with 100 units/ml of fresh S1 nuclease. Autoradiography was done overnight.

per one chicken genome (Fig. 4a, lane 3). Following hybridization in the gel (Fig. 4b) the bands visible in Fig. 4a become much more pronounced and a number of previously undetectable bands appears (Fig. 4b, lanes 2,3,5,6,8,9). In order to achieve additional enrichment for repeated DNA fragments relative to single copy sequences, the gel was subjected to a second cycle of hybridization following S1 nuclease digestion, i.e. DNA in the gel was again denatured, renatured and treated with S1 nuclease. As a result of this treatment, the intensity of the bands relative to the background was drastically increased (Fig. 4c). Most of the bands can be seen only in the lanes with the highest amount of driver (lanes 3,6,9) due to significant losses of DNA after two cycles of hybridization. In the digest of chicken DNA, 24 bands of various intensity corresponding to repeated EcoRI fragments ranging in size from 18 to 1.15 kb could be identified (Fig. 4c, lane 3). Most of these bands have not been detected in the previous studies on repetitive sequences in chicken DNA. λ DNA fragments, added at the ratio of 100 λ DNA per chicken genome, were visible as very strong bands, exceeding in their intensity most of the bands corresponding to repeated fragments of chicken DNA (Fig. 4c, lane 9). At the ratio of 15 λ DNA per chicken genome, detection of the weak bands corresponding to the EcoRI fragments of λ DNA is complicated by co-migration of some of these fragments with the repeated fragments of chicken DNA. However, at least some of the λ DNA fragments, such as the 3.38 kb band, are sufficiently separated from the chicken DNA fragments to be identified in Fig. 4c, lane 6 by comparison with the chicken DNA pattern (lane 3). 15 copies therefore seems to be close to the limit of detection for an amplified chicken DNA fragment at the present time.

The strong appearance of λ DNA fragments in the mixture of 100 λ DNA per chicken genome should not be taken to indicate that most EcoRI fragments of chicken DNA detectable by this procedure are repeated less than 100 times. For example, the 7.9 kb fragment of chicken DNA (indicated with an arrow in Fig. 4b, c) corresponds in its size to the reported EcoRI fragment containing chicken ribosomal DNA (21) and may therefore represent a part of the chicken ribosomal gene cluster. Ribosomal DNA sequences have been shown to be present at 200-240 copies per haploid genome (22), but the 7.9 kb band is much weaker than the bands corresponding to 100 copies of λ DNA. This apparent contradiction can be explained by considering that divergence between the repeat units of chicken ribosomal genes, particularly in the spacer region, results in more diffused bands and less precise sequence homology than in the bands formed by completely homogeneous λ DNA fragments. As a result, the
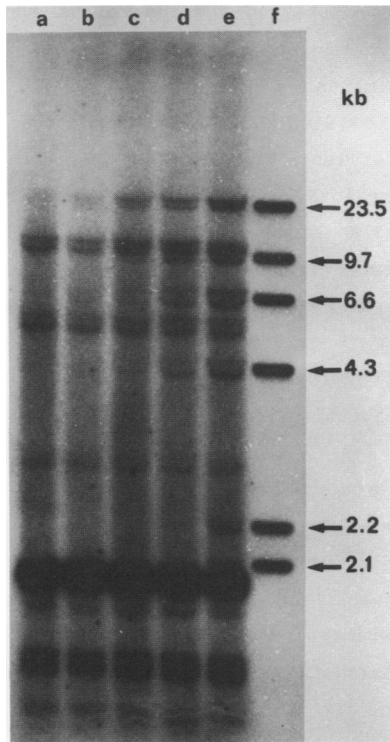
Fig. 5.   Detection of repeated HindIII fragments in DNA from a human
lymphocyte line and in mixtures of human and λ phage DNAs. Human DNA (lane a)
and mixtures of human and λ DNA at genomic ratios of 1 human: 15 λ DNA (lane
b), 1 human: 30 λ DNA (lane c), 1 human: 50 λ DNA (lane d) and 1 human: 100 λ
DNA (lane e) were digested with HindIII and tracer DNA was prepared as
described in the legend to Fig 4. Each lane contains 10^7 dpm of tracer DNA
and 12 μg of the same unlabeled driver DNA. HindIII fragments of λ DNA
(10,000 dpm in 0.6 μg) were used as size standards (lane f).   After
electrophoresis in 1% agarose, the gel was subjected to two cycles of
hybridization in 5 x SSPE/ 50% formamide at 45°C. The time of hybridization
was two hours for the first cycle and 10 hours for the second cycle.   100
units/ml S1 nuclease were used in both cycles.   Autoradiography was done
overnight.

local concentration of DNA in the gel and the efficiency of hybridization are

expected to be much higher for λ DNA fragments than for the fragments of the

ribosomal gene cluster or any other repeated DNA sequences that have

accumulated some degree of sequence divergence in the course of evolution.

       The results of the experiment shown in Fig. 4 suggest that the repeated

fragments in eukaryotic DNA are best detected when the maximum possible amount

of driver DNA is loaded on the gel, and the gel is subjected to two cycles of hybridization. In order to determine the sensitivity of this technique for precisely repeated fragments of mammalian DNA, DNA prepared from a human lymphocyte line was mixed with various amounts of λ phage DNA and digested with HindIII. 10^7 dpm of labeled tracer DNA together with 12 μg of the same unlabeled driver DNA were loaded into each lane, and after electrophoresis DNA in the gel was subjected to two cycles of hybridization (Fig. 5). It should be noted that some repeated fragments that are normally present in human DNA are underrepresented in this experiment (lane a) due to the loss of repeated sequences during in vitro growth of human cells (23 and our unpublished results). The HindIII fragments of λ phage DNA could be detected in lanes c, d and e, containing respectively 30, 50 and 100 copies of λ phage DNA per haploid amount of human DNA (2.9 x 10^9 kb), but not in lane b, containing 15 copies of λ DNA per human genome. Therefore, the present limit of detection for a precisely repeated fragment of mammmalian DNA is about 30 copies per haploid genome.

The presence of amplified DNA in MTX-resistant mouse cell lines was demonstrated in the experiment shown in Fig. 6. MTX-resistance in these lines results from amplification of the gene coding for dihydrofolate reductase (DHFR), the target enzyme for MTX (24). Various amounts of flanking sequences are co-amplified with the DHFR gene in different lines (25,26). DNA from different cell lines was digested with Bam HI. ^{32}P-labeled tracer DNA was mixed with 10-12 μg of the appropriate driver DNA, electrophoresed in a 1% agarose gel and subjected to two cycles of hybridization in the gel. DNA in lane d was isolated from R500, a subline of 3T3 cells resistant to 250 μm MTX (27) and containing 170 copies of the DHFR gene per haploid genome (R.M. Snapka and A. Varshavsky, unpublished results). Comparison of the pattern of repeated Bam HI fragments in lane d and lane a, which contains DNA from the MTX-sensitive 3T3 cells, reveals a large number of amplified fragments in R500 DNA (indicated with arrowheads). Lanes b and c contain mixtures of 3T3 and R500 DNA corresponding to either 20 (lane b) or 50 (lane c) copies of the DHFR gene per haploid genome. The amplified fragments of R500 DNA are readily detectable in lane c, but not in lane b. Assuming that the degree of amplification of these fragments corresponds to that of the DHFR gene, the sensitivity of this experiment (between 20 and 50 copies per genome) is in good agreement with the results of the reconstruction experiment in Fig. 5 (about 30 copies per genome).

Lane f in Fig. 6 contains DNA from R.3 cells, a subline of 3T6 cells
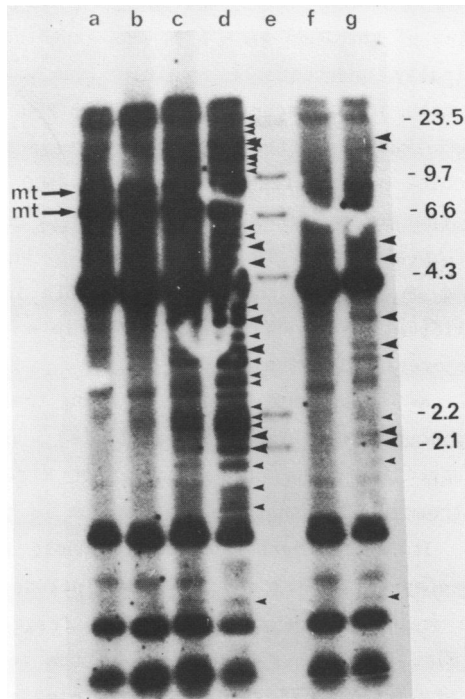
Fig. 6. Detection of amplified Bam HI fragments in MTX-resistant mouse cell lines. DNA was digested with Bam HI and tracer DNA was ^{32}P-labeled as described in Materials and Methods. After electrophoresis in 1% agarose, the gel was subjected to two cylces of hybridization in 3 x SSPE/ 50% formamide at 37°C. The time of hybridization was two hours for the first cycle and 11 hours for the second cycle. The concentration of S1 nuclease was 50 units/ml in the first cycle and 100 units/ml in the second cycle. Autoradiography was done overnight.

In lanes a-f the same DNA perparations were used both as a tracer and as a driver. Lane a contains DNA from MTX-sensitive NIH 3T3 cells (1 copy of the DHFR gene per haploid genome) and lane d contains DNA from MTX-resistant R500 cells (170 copies of the DHFR gene). Lanes b and c contain mixtures of 3T3 and R500 DNA corresponding to either 20 copies (lane b) or 50 copies (lane c) of the DHFR gene per haploid genome. Lane f contains DNA from the nuclei of an MTX-resistant line R.3 (16 copies of the DHFR gene). Lane g contains R.3 DNA as a tracer and R500 DNA as a driver. The amount of tracer DNA was 1 μg in lanes a-d and 0.5 μg in lanes f and g. The amount of radioactivity in the tracer was 10^7 dpm (lanes a and b), 9 x 10^6 dpm (lane c), 4.3 x 10^6 dpm (lane d) and 2.1 x 10^6 dpm (lanes f and g). The amount of driver DNA was 12 μg (lanes a-c, f) or 10 μg (lanes d and g). HindIII fragments of λ DNA (3,000 dpm in 0.6 μg) were used as size standards (lane e).

Arrowheads indicate amplified DNA fragments that are not present in 3T3 DNA. Large arrowheads: the putative fragments of the DHFR gene and its immediate flanking regions (see text). mt: Bam HI fragments of mitochondrial DNA (29 and our unpublished results).

that was independently selected for resistance to 0.3 μm MTX and contains approximately 16 copies of the DHFR gene (7). As expected, the amplified DNA fragments are practically undetectable in this lane. However, when ^{32}P-labeled R.3 DNA was used as a tracer and unlabeled R500 DNA was used as a driver (lane g), hybridization in the gel revealed several additional bands, corresponding to a subset of amplified fragments in R500 DNA (lane d). This result suggests that the genomes of R.3 and R500 cells contain non-identical but overlapping sets of amplified Bam HI fragments. While these fragments could not be detected in lane f, they become visible in lane g, because a higher degree of amplification in R500 DNA resulted in a higher efficiency of hybridization for the overlapping fragments.

The results of the experiment in Fig. 6 show that two independently derived MTX-resistant mouse cell lines have amplified a common subset of DNA sequences, thereby suggesting a common mechanism for MTX-resistance in these two lines. It is already known that this mechanism is the amplification of the DHFR gene (24). It would therefore be reasonable to suggest that the commonly amplified sequences in R.3 and R500 DNA include the DHFR gene. In fact, the sizes of several commonly amplified Bam HI fragments (indicated with large arrowheads in Fig. 6) roughly correspond to the reported sizes of the Bam HI fragments of the mouse DHFR gene and its immediate flanking regions (28). Therefore, one can speculate that these fragments actually contain the DHFR gene. Additional experiments, which are outside the scope of the present article, would be required to establish this fact. It should be noted, however, that no band corresponding to the largest (>20.5 kb) Bam HI fragment of the DHFR gene (28) could be detected among commonly amplified fragments. This is probably due to the above mentioned problem of low sensitivity of the technique for the high molecular weight DNA fragments.

DISCUSSION
        The technique of DNA renaturation in agarose gels provides the basis for a general strategy for identification and characterization of amplified DNA sequences in eukaryotic DNA. Genomic DNA from a cell population which is assayed for gene amplification is digested with a restriction enzyme and reassociated in the gel next to a similar digest of genomic DNA from control cells. Amplified DNA fragments are visualized as a series of bands that are not present in control DNA. The finding that the homogeneous λ DNA sequences appear to reassociate in the gel more readily than the relatively heterogeneous repeated fragments of eukaryotic DNA suggests that recently

amplified DNA sequences are preferentially detectable by this technique as compared to more ancient repetitive sequences. This procedure also allows the comparison of independently amplified DNA sequences in different cell populations. When DNA from one cell population is used as a tracer, and DNA from another population is used as a driver in the experiments of the type illustrated in Fig. 6, only those restriction fragments that have been amplified in both cell populations would be detectable. These commonly amplified fragments, that are likely to contain the gene(s) responsible for the particular cell phenotype or to be located in the vicinity of such a gene, can be subsequently cloned and analyzed. This strategy has been recently successfully used to detect amplified DNA sequences and to determine the common sequences that have been amplified in two independently derived hamster cell lines that are simultaneously resistant to several different cytotoxic drugs (I.B.R., A. Varshavsky, H. Abelson, D. Housman and N. Howell, manuscript in preparation). Another approach to identification of those amplified fragments that contain transcribed genes consists of transferring unlabeled DNA onto nitrocellulose filters (1) following two cycles of renaturation in the gel. The filters are then hybridized to a cDNA probe prepared from total mRNA extracted from the cells that contain amplified DNA. Since single copy sequences have been removed from the gel by S1 nuclease, hybridization of repeated and amplified fragments to cDNA is readily detectable (E. Rose, I.B.R., M. Murray, H.N. Munro and A. Varshavsky, unpublished results).

The method described in this article provides a useful approach for several other types of studies, such as analysis of the structure and variety of long repetitive sequences in eukaryotic DNA or characterization of evolutionarily conserved sequences in different groups of organisms. Extended regions of homology, detectable by this method, can be analyzed not only between different genomes, but also between isolated chromosomes, extrachromosomal DNA, or integrated and unintegrated forms of viral DNA. In another possible application of this technique, the use of a genomic clone as a tracer and of chromosomal DNA as a driver allows one to detect size alterations in the corresponding region of the genome. In this type of experiment, as opposed to Southern (1) hybridization, the presence of repetitive sequences within the genomic clone should not affect the specificity of hybridization.

The finding that S1 nuclease is able to digest single-stranded DNA in agarose gels can have other applications aside from the one used here. For example, it may be possible to analyze S1 nuclease-sensitive regions in a

mixture of DNA or chromatin fragments by two-dimensional mapping, using S1 nuclease in situ digestion after separation in the first dimension.

While the procedure described in this article allows one to identify repeated and amplified DNA fragments as bands in an autoradiogram, it also results in substantial purification of such fragments, and therefore can probably be used as a preparative as well as analytical method.

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