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Differential cloning of genomic DNA: Cloning of DNA with an altered primary structure by in-gel competitive reassociation

(rearrangement/amplification/mammalian genomes)

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ABSTRACT **A procedure was developed for cloning (anonymous) DNA sequences whose primary structures differ between two DNA samples. The procedure is based upon in-gel competitive DNA reassociation after electrophoresis of a mixture of restriction enzyme-digested target DNA (from which clones are to be isolated) and a large excess of unclonable reference DNA (competitor DNA). Inclusion of polyethylene glycol in the reassociation buffer greatly improved the in-gel reassociation efficiency, which was critical for the practical use of the procedure. Using this technique, we obtained several clones from rat brain (target) DNA, which may have been derived from tissue (brain)-specific altered DNA structures. The details of this procedure and its possible applications are discussed.**

Mammalian genomes are organized into complex arrays of unique genes and repetitive DNA sequences, which occupy a considerable portion of the total DNA. A number of methods are currently available for cloning genomic DNA sequences of interest, which include the use of DNA hybridization with appropriate DNA probes. Consider the situation, however, where there are two DNA preparations from higher organisms differing only in their primary structure at a limited number of unidentified sites as a result of DNA modification, rearrangement, or amplification. When one wants to clone a DNA fragment containing the altered structure, no efficient general procedures are currently available unless the sequences are completely absent in one of the preparations due to deletion or are present in only one due to association with specific chromosomes such as sex chromosomes.

General procedures for cloning genomic DNA with a rearranged or amplified structure of unknown origin (differential cloning of genomic DNA), once established, would be quite useful for studying and identifying possible causes of a variety of biological phenomena. Here, as a first step toward this goal, we report a procedure for cloning DNA restriction fragments with such altered structures. The procedure is based upon in-gel competitive reassociation between target DNA fragments (from which clones are to be isolated) and reference DNA fragments (competitor DNA). We also present the results of analysis of clones obtained through this procedure from rat brain DNA (target DNA) upon competition with liver DNA (reference DNA) of the same animal.

MATERIALS AND METHODS

Materials. Restriction enzymes, kinases, and bacterial phosphatase were purchased from Takara Shuzo (Kyoto, Japan). Proteinase K, RNase A, and Hoechst dye 33258 were supplied by Sigma. Polyethylene glycol (PEG) 8000 (lot no. B17707, previously PEG 6000) and agarose (SeaKem GTC) were purchased from Baker and FMC, respectively. Radioisotopes and random priming kits were obtained from ICN and Boehringer Mannheim, respectively. All the agents used were of reagent grade. Rats were obtained from Nippon Bio-Supply Center (Tokyo).

Isolation of DNA. Tissue DNA was isolated by the conventional phenol procedure. Tissue that had been removed from a rat (Wistar, 8-week-old female) under anesthesia with chloroform was homogenized with a Dounce homogenizer in 10 mM Tris·HCl, pH 8.0/10 mM EDTA/100 mM NaCl (10 ml/g of tissue). SDS and proteinase K were then added at concentrations of 0.5% and 0.2 mg/ml, respectively. The samples were incubated at 37°C for 18 hr and treated with an equal volume of phenol three times. The aqueous phase was dialyzed for 18 hr against TE buffer (10 mM Tris·HCl, pH 7.5/1 mM EDTA), and the dialyzed samples were treated with RNase A (2 μg/ml for 1 hr at 37°C) followed by proteinase K (100 μg/ml) in the presence of SDS (0.5%) for 18 hr at 37°C. The samples were treated with phenol as before three more times, and the aqueous phase was treated twice with chloroform/3-methyl-1-butanol, 24:1, to remove residual phenol. DNA was then precipitated with 2.5 volumes of ethanol and dissolved in 10 mM Tris·HCl, pH 7.5/1 mM EDTA. *Bacillus subtilis* ϕ105 DNA prepared by the conventional phenol procedure was kindly provided by F. Kawamura of this Institute. The DNA concentration in each sample was determined by the fluorometric method (1), which measures fluorescence developed in the presence of Hoechst 33258 [2′-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1*H*-benzimidazole hydrochloride], using a Hitachi F2000 fluorescence spectrometer (excitation at 356 nm, emission at 458 nm). Calf thymus DNA (Sigma) was used as a DNA standard.

In-Gel Competitive Reassociation. Brain DNA (target DNA) and liver DNA (reference DNA) from the same rat (Wistar, 8-week-old female) were digested with *Eco*RI to completion. The digest from the liver was further treated (dephosphorylated) with bacterial alkaline phosphatase (5.25 units per 100 μg of DNA) for 2 hr at 65°C, and the DNA was precipitated with ethanol and dissolved in TE buffer at 2 μg/μl (2). One (or two) microgram of the brain DNA fragments was mixed with 100 μg of the dephosphorylated liver DNA fragments, and the mixture (≈50 μl) was loaded onto an agarose [1.2% (wt/vol), SeaKem GTG, FMC] gel (6 mm thick), which gave a cross section of ≈50 mm^2 for electrophoresis of the DNA. The dimension of a combed well was 1 mm deep × 14 mm wide (× 6 mm thick). For smaller scale experiments, a smaller well (1 mm × 4 mm) was employed. After electrophoresis at 20 V for 17 hr in TAE buffer (40 mM Tris acetate, pH 7.8/1 mM EDTA), the gel was placed in

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Abbreviation: Amp^r, ampicillin resistant.

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alkaline solution (0.5 M NaOH/0.6 M NaCl) for 30 min at room temperature with gentle shaking. This process was repeated once more with fresh alkaline solution. The gel was then rinsed twice with distilled water and incubated in reassociation buffer [50% (vol/vol) formamide/25 mM sodium phosphate buffer, pH 6.8/1 M NaCl/5 mM EDTA/10% (wt/vol) PEG 8000] for 20 min at room temperature. After repeating the incubation three times, each time with fresh reassociation buffer, the gel was incubated in the same reassociation buffer for 17 hr at 45°C. After incubation, the gel was washed twice for 20 min at room temperature in TE buffer (10 mM Tris·HCl, pH 7.5/1 mM EDTA). A portion of the gel where 0.5- to 2.0-kilobase (kb) DNA fragments migrated was cut out with a razor blade, and DNA was recovered from the gel with Geneclean (Bio 101, La Jolla, CA).

The DNA was ligated to *Eco*RI/bacterial alkaline phosphatase-treated pUC13 (Pharmacia; pUC13 *Eco*RI/BAP) by using a ligation kit (Takara Shuzo). Usually 1.5 μg of the linearized and dephosphorylated pUC13 was used for 1 μg of the DNA recovered from the gel for the ligation, unless otherwise specified. Competent *Escherichia coli* (DH5α) cells were transfected with the ligated DNA as originally described by Cosloy and Oishi (3) and as modified by Hanahan (4). Before transfection, the competent cells were tested to ensure that at least one ampicillin-resistant (Amp^r) transfectant was produced per 1 pg of control pUC13 DNA to obtain maximum complexity of the clones. After mixing 100 ng of the ligated DNA with 5×10^9 competent DH5α cells, the cells were incubated for 1 hr for expression and overnight in the presence of ampicillin (50 μg/ml). Plasmid DNA was extracted from the cells by the alkaline procedure (5) and further purified by ethidium bromide/CsCl centrifugation. Usually 20–30% of the closed circular DNA thus isolated contained inserts. The cloned DNA mixture was used as target DNA for the second round of in-gel competitive reassociation (see below), and the whole process (see above) was repeated again.

Southern Hybridization. Restriction enzyme-digested DNA samples (10–20 μg each) were electrophoresed on agarose (1.4% wt/vol) gels, transferred to nylon membranes (Pall Biodyne), and hybridized with ^{32}P-labeled probes (30 ng in 10 ml of hybridization buffer, unless otherwise specified) according to the procedure described by Southern (6). After hybridization, the membranes were washed twice with 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0), twice with 0.5× SSC, and twice with 0.1× SSC (all at 65°C for 30 min). All the SSC solutions used for the washes contained SDS (0.1%). The filters were then autoradiographed (18–36 hr at −80°C) using Kodak XAR film. The probes were random primed with [α-^{32}P]dCTP (specific activity = 3000 Ci/mmol; 1 Ci = 37 GBq) by using a priming kit (7).

RESULTS

Principle and Outline of the In-Gel Competitive Reassociation. Two genomic DNA preparations, target DNA from which clones are to be isolated and reference DNA serving as competitor DNA, are treated with any (but the same) restriction enzyme that can produce cloning sites at the termini of the products. One of the DNA samples (reference DNA) is further treated with alkaline phosphatase to eliminate terminal phosphates, thus making the DNA nonclonable (see below). The target DNA is mixed with a large excess of the reference DNA (for example, at a target DNA-to-reference DNA ratio of 1:100), and the mixture is electrophoresed through an agarose gel. The DNA is then alkaline-denatured and reassociated (renatured) in the gel. Under these conditions, when the two DNA samples are identical with respect to their restriction enzyme cleavage sites (thus producing the same restriction fragments), the majority (99%) of denatured restriction fragments of the target DNA will reassociate with the excess amount of corresponding unclonable reference DNA fragments that have migrated to the same position (in-gel competitive reassociation). On the other hand, if the target DNA preparation contains a DNA fragment lacking a counterpart reference DNA fragment at the same migration position, the fragment will reassociate only with itself, if the C_0t value for the fragment is sufficiently high for the reassociation.

Whole DNAs are then extracted from the gel and cloned into a linearized and dephosphorylated plasmid vector. Only reassociated target DNA fragments that have phosphorylated termini for both strands will be cloned into the vector. After transfection in *E. coli*, the plasmid DNA is isolated from the bacteria. The DNA is used again as target DNA and the whole procedure is repeated. If the ratio of each fragment of target DNA to that of reference DNA is 1:100 in the competition mixture, a 10,000-fold (100 × 100) concentration of unique target DNA fragments is theoretically expected after two cycles of in-gel competitive reassociation.

Optimization of In-Gel Reassociation. One major hurdle to clear in realizing the principle described above was how to increase the efficiency of in-gel reassociation. In preliminary experiments, when 10 μg of rat genomic (brain) DNA (1 μg/mm^2 cross section) was electrophoresed (without reference DNA) through a 1.2% agarose gel and subjected to in-gel reassociation under the conventional reassociation (renaturation) conditions (for example, 50% formamide/25 mM sodium phosphate buffer, pH 6.8/1 M NaCl/5 mM EDTA), only 2–4% of the DNA, compared to the original DNA that was not subjected to denaturation and reassociation, was recovered as transfectable clones (data not shown; see also Fig. 1). Among a variety of conditions examined to increase the in-gel reassociation efficiency, we found that the presence of PEG 8000 in the reassociation buffer greatly increased the in-gel reassociation of genomic DNA as assayed by the recovery of transfectable DNA clones after reassociation. Fig. 1 shows the effect of PEG 8000 during in-gel reassociation of denatured rat genomic (brain) DNA on the recovery of transfectable DNA clones as a function of PEG concentration. It is quite clear that, at 10% (wt/vol) PEG 8000, the proportion of transfectable, reassociated DNA reached ≈40% of the original DNA after 17 hr of incubation at 45°C (Fig. 1). The reason for the effect of PEG 8000 on the in-gel reassociation was not clear. We found, however, that PEG 8000 blocked the formation of DNA aggregates, apparently produced by repetitive DNA sequences in the genomic DNA, which seemed to contribute to the low recovery of transfectable clones in the absence of PEG. Other polymers, including dextran sulfate, were not as effective as PEG 8000 (data not shown).

Fig. 2 shows the time course of the reassociation (Fig. 2*A*) and the effect of concentration of genomic DNA in the gel on the reassociation (Fig. 2*B*). The reassociation (assayed by transfection) reached a maximum level at 20 hr of incubation (at 45°C) (Fig. 2*A*) and at ≈500 ng of genomic DNA per mm^2 (electrophoretic cross section) of the gel (Fig. 2*B*). Based upon these results, we decided to employ the following conditions for the reassociation of genomic DNA in agarose gels: incubation in reassociation buffer (50% formamide/25 mM sodium phosphate buffer, pH 6.8/1 M NaCl/5 mM EDTA/10% PEG 8000) for 17–20 hr at 45°C with a target DNA concentration of at least 40 ng/mm^2.

In-Gel Competitive Reassociation. To verify the experimental results described above, we attempted to clone genomic DNA sequences that might exhibit different restriction patterns depending upon their tissue sources, although tissue-specific rearrangement or amplification of mammalian ge-

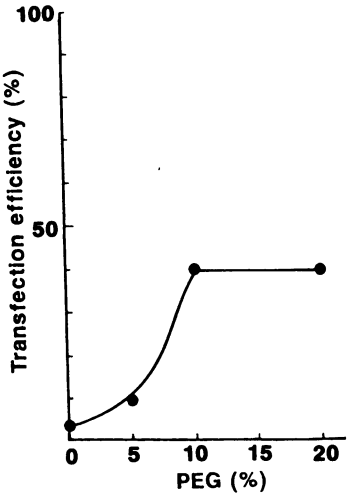

FIG. 1. Effect of PEG concentration on in-gel reassociation of rat genomic DNA. *Eco*RI-digested rat brain (Wistar, 8-week-old female) DNA (10 μg in 10 μl) was electrophoresed through an agarose gel (1.2% wt/vol) (10 mm^2 of electrophoretic cross-section). The dimensions of the well were 1 mm × 4 mm. The DNA in the gel was denatured and reassociated for 17 hr at 45°C in reassociation buffer containing various concentrations (wt/vol percentages) of PEG 8000 as indicated along the abscissa. The DNA (0.5–2 kb) was recovered from the gel, ligated to a linearized and dephosphorylated vector (pUC13), and used as donor DNA for transfection of *E. coli*. Amp^r transfectants were counted, and the transfection efficiency (%) was calculated from the ratio of Amp^r transfectants to those obtained with an equal amount of control DNA that was electrophoresed but not subjected to denaturation and reassociation.

nomes has not yet been reported except for gene rearrangement in lymphocytes (8–11) and DNA amplification in a number of drug-resistant cells as well as tumor cells. We also monitored the concentration of an artifically added DNA fragment (as an intrinsic standard DNA) after each step of in-gel competitive reassociation. DNA was isolated from the brain and liver of the same rat (Wistar, 8-week-old female) and used as target (brain) and reference (liver) DNA. The two DNA preparations were digested with *Eco*RI to completion, and the reference (liver) DNA was further treated with alkaline phosphatase. Two micrograms of brain DNA, 100 μg (50-fold excess) of the liver DNA, and 450 pg (equivalent to ≈10 copies per rat genome) of *Eco*RI-digested total *B. subtilis* phage ϕ105 DNA (genome size of 36.6 kb) as an intrinsic standard DNA were then mixed and electrophoresed through an agarose gel. After electrophoresis, the gel was soaked in alkaline solution to denature the DNA and then incubated in the reassociation buffer for 17 hr at 45°C. The portion of the gel that contained DNA fragments of 0.5–2.0 kb was then cut out, and the DNA was extracted from the gel. The DNA was then ligated to a vector (pUC13), which had been treated with *Eco*RI and alkaline phosphatase, and used to transfect *E. coli* (for details, see *Materials and Methods*). As had been expected, the alkaline phosphatase treatment of the vector DNA reduced the cloning efficiency to less than one-thousandth of that with untreated control pUC13 vector (data not shown).

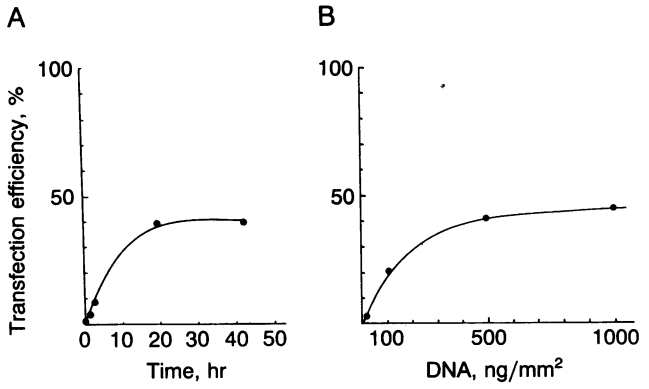

FIG. 2. Efficiency of in-gel reassociation as a function of incubation time (*A*) and DNA concentration (*B*). (*A*) *Eco*RI-digested rat brain (Wistar, 8-week-old female) DNA (10 μg in 10 μl) was electrophoresed, denatured, and reassociated in the gel. At different time intervals, DNA (0.5–2 kb) was recovered from the gel and ligated to a linearized and dephosphorylated vector (pUC13). The DNA was then used as donor DNA for transfection of *E. coli*. Amp^r transfectants were counted, and the transfection efficiency (%) was calculated from the ratio of Amp^r transfectants to those obtained with an equal amount of control DNA that was electrophoresed but not subjected to denaturation and reassociation. (*B*) Same as *A*, except that *Eco*RI-digested rat brain DNA at different concentrations was electrophoresed, denatured, and reassociated (for 17 hr at 45°C). The numbers on the abscissa show the amount of DNA (ng) per mm^2 of the electrophoretic cross section.

For the second round of in-gel competitive reassociation, the total plasmid DNA recovered from the Amp^r transfectants was used as target DNA after treatment with *Eco*RI. For this, DNA equivalent to ≈10 ng of insert DNA was mixed with 100 μg of reference (liver) DNA. After repeating the whole procedure (electrophoresis, denaturation, reassociation, ligation, and transfection), the DNA recovered from *E. coli* was subjected to further analysis as described below. We employed 10,000-fold excess (in DNA weight) of competitor DNA for the second round (10 ng of target DNA obtained after the first round versus 100 ng of genomic DNA). The actual competition between the two DNA samples in the second round, however, should vary depending upon the degree of enrichment in the first round, the complexity of DNA clones obtained after *E. coli* transfection, the proportion of DNA fragments of 0.5 to 2.0 kb among the total genomic DNA, and other factors.

Concentration of a Standard ϕ105 DNA Fragment. We first compared the concentration of a fragment of standard ϕ105 DNA present in DNA after each step of in-gel competitive reassociation. An equal amount of the starting DNA [a mixture of brain DNA, reference (liver) DNA, and standard ϕ105 DNA], the once-cycled DNA, and the twice-cycled DNA were digested with *Eco*RI, electrophoresed, and probed with the ^{32}P-labeled 1.1-kb *Eco*RI fragment of ϕ105 DNA. As shown in Fig. 3*B*, the intensity of the band that hybridized with the 1.1-kb ϕ105 fragment increased considerably after each step of in-gel competitive reassociation. From the intensity of the standard reference DNA (Fig. 3*A*), we estimated the extent of concentration after two cycles of in-gel competitive reassociation to be ≈3000-fold. Later experiments indicated that five or more copies of the ϕ105 DNA per genome were enriched after repeated cycles of the competitive reassociation. Thus, the competitive in-gel reassociation procedure was quite useful for concentrating unique DNA fragments if present as several copies or more per genome.

Analysis of Clones Obtained from Two-Cycle In-Gel Competitive Reassociation. We examined DNA clones from Amp^r transfectants obtained with the twice-cycled rat target (brain) DNA. Among 750 randomly selected Amp^r transfectants, 257 clones contained inserts. As shown in Table 1, 30 clones (11.6%) had inserts with highly or middle/low repetitive DNA sequences, and the rest (227 clones, 88.4%) contained inserts representing single (or low) copy sequences. Thus, after two cycles of in-gel competitive reassociation, the proportion of repetitive DNA sequences among the total DNA, originally accounting for 30% or more of the DNA, was reduced to ≈10%. The reason for the disproportional drop in the number of repetitive sequences is not clear.

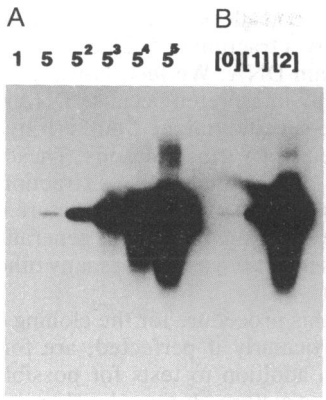

FIG. 3. Concentration of a standard DNA fragment (ϕ105, 1.1 kb of DNA) by in-gel competitive reassociation. (*A*) *B. subtilis* ϕ105 phage DNA was digested with *Eco*RI and 0.8 pg (lane 1), 4 pg (lane 5), 20 pg (lane 5^2), 100 pg (lane 5^3), 500 pg (lane 5^4), and 2500 pg (lane 5^5) of the digested DNA was electrophoresed in an agarose gel (1.4%). The DNA was transferred to a nylon membrane filter and probed with the ^{32}P-labeled 1.1-kb *Eco*RI fragment of ϕ105 DNA. (*B*) *Eco*RI-digested rat brain (Wistar, 8-week-old female) DNA (2 μg) was mixed with 100 μg of *Eco*RI-digested and dephosphorylated liver DNA of the same animal and 450 pg of *Eco*RI-digested *B. subtilis* ϕ105 phage DNA. The mixture (53 μl) was subjected to agarose gel electrophoresis (53 mm^2 electrophoretic cross section) and in-gel competitive reassociation. DNA with molecular sizes of 0.5–2.0 kb was recovered from the gel and ligated to linearized and dephosphorylated pUC13. After transfection in *E. coli*, plasmid DNA was extracted and purified (once-cycled DNA). For the second round of in-gel competitive reassociation, the plasmid DNA (equivalent to $\approx$10 ng of insert DNA) was mixed with 100 μg of the *Eco*RI-digested and dephosphorylated rat liver DNA, and the whole process was repeated (twice-cycled DNA). The DNA (10 μg each) from each step (before in-gel competitive reassociation, once-cycled DNA, and twice-cycled DNA) was electrophoresed in agarose gel (1.4%) and subjected to Southern hybridization using the same ^{32}P-labeled ϕ105 1.1-kb DNA fragment as used for *A* as a probe. Lanes: 0, DNA before in-gel competitive reassociation; 1, once-cycled DNA; 2, twice-cycled DNA.

Two of the three classes of clones shown in Table 1, clones with highly repetitive sequences or low/single copy sequences, were further classified into groups according to the size of the inserts: 3 groups for highly repetitive DNA and 5 groups for single/low copy DNA. Members of each group were then examined for their ability to cross-hybridize with other members of the same group. We found that some of the inserts in the same molecular size group cross-hybridized with each other, suggesting that they were derived either from repetitive sequences or from the same genomic fragments but enriched accidentally or as a result of in-gel competitive reassociation. We have so far identified 14 cross-hybridizing groups (2 for highly repetitive DNA and 12 for single/low copy DNA) with a total of 128 group members, which range from 3 to 18 (members) per cross-hybridizing group. The rest of the clones do not belong to any of these groups and probably represent independent clones or belong to unidentified cross-hybridizing groups with small numbers of group members.

Table 1. Analysis of clones with inserts obtained after twice-cycled in-gel competitive reassociation

Type of DNA sequence	Number of clones (%)
Highly repetitive	20 (7.7)
Middle/low repetitive	10 (3.9)
Low/single copy	227 (88.4)
Total	257 (100.0)

Classification of highly repetitive, middle/low repetitive, and low/single copy was done after colony hybridization of each clone with ^{32}P-labeled total genomic rat DNA.

We analyzed representatives from several (two repetitive and four single-copy sequences) groups containing the highest numbers of cross-hybridizing clone members and found that three of them yielded apparently different Southern hybridization patterns between *Eco*RI-digested brain and liver DNA when used as probes. One of them (termed BL-1 with 12 similar clones) represented a repetitive DNA sequence and two of the others (termed clone 135 and clone 190 with 18 and 15 similar clones, respectively) contained inserts with apparently low/single copy sequences.

Sequencing of the BL-1 insert (621 base pairs) indicated that it is a member of the LINE family, one of the highly repetitive mammalian sequences that exists at over 10^4 copies per genome; BL-1 represents a portion of the distal putative open reading frame with a possible reverse transcriptase function (12, 13). The sequence homology of the fragment with that of the published LINE sequence (LINE 3) (14) was $\approx$97%. We found, however, that the sequences of all 12 clones of BL-1 were identical, suggesting that they were derived from the same LINE family member. We examined *Eco*RI-digested brain and liver DNA isolated from the same rat (Wistar, 8-week-old male) by Southern blot analysis with BL-1 as a probe under stringent washing conditions after gel electrophoresis. As shown in Fig. 4, a discrete band of 0.6 kb, equivalent to $\approx$100 copies of BL-1 per genome, was observed only with brain DNA, regardless of species, sex, or age of rats. Details of the analysis of BL-1, characterized by possible tissue (brain and lens)-specific amplification during the developmental process, were published recently (15).

Clone 135 contained a fragment of 580 base pairs, which seemed to be derived from a low/single copy DNA sequence. Southern blot hybridization using clone 135 as a probe with brain and liver DNA from the same rat (Wistar, 8-week-old male) showed that there were approximately 5-fold more copies per genome in brain compared to liver at least in this particular rat (data not shown). The third clone analyzed (clone 190) had an insert of $\approx$450 base pairs. Clone 190 gave an apparently amplified pattern with brain DNA similar to that observed with clone 135 (data not shown). The apparent amplification was $\approx$4- to 5-fold. Precautions in interpreting the results are mentioned below.

DISCUSSION

In this paper, we have reported a procedure that was designed for the cloning of unidentified genomic DNA with an altered primary structure as a result of DNA rearrangement (including deletion, insertion, and inversion) or amplification. The essence of the procedure is the use of in-gel reassociation in the presence of excess amounts of unclonable reference DNA, which serve as a competitor for cloning.

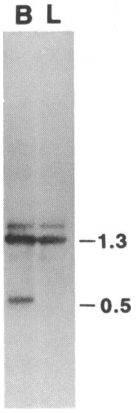

FIG. 4. Southern blot analysis of rat brain and liver DNA with the BL-1 probe. DNA samples (10 μg each), which had been isolated from brain and liver of a rat (Wistar, 8-week-old female), were digested with *Eco*RI and subjected to agarose (1.4%) gel electrophoresis. After transfer to a membrane, the DNA was hybridized with ^{32}P-labeled BL-1 (3 ng/20 ml), and the filter was exposed to an x-ray photographic film. B, brain DNA; L, liver DNA. Numbers at the right indicate the molecular size of DNA in kb.

We overcame the major obstacle for in-gel reassociation, the low efficiency of reassociation, by including PEG 8000 in the reassociation buffer. The presence of PEG also seemed to minimize the interfering effect of repetitive DNA present in mammalian genomes on legitimate DNA–DNA reassociation, which apparently resulted from aggregation of DNA fragments in the gel. This, along with the combination of several other parameters (temperature, pH, ionic strength, and formamide concentration), improved for this purpose, made it possible to attain a reassociation efficiency as high as 40% of the genomic DNA fragments (assayed by transfection in *E. coli*) with practical amounts of DNA loaded onto the gel. This seemed to be adequate, although possibly not optimal, for some of the experiments for which the procedure was originally intended. After two cycles of in-gel competitive reassociation, the intrinsic standard DNA fragment (ϕ105, 1.1 kb) was concentrated ≈3000-fold. We obtained the same level of concentration when at least five copies (per genome) of the standard DNA fragment were present in the original sample, but cloning of a single-copy fragment (per genome) with an altered primary structure was not always reproducible (data not shown), suggesting that C_0t values at these DNA concentrations (between one and five copies per genome) in the gel under the reassociation conditions employed here were at a level quite critical for complete reassociation. Reproducible cloning of an altered sequence existing as one copy per genome or less would require a further improvement in the reassociation conditions or repeated cycles (more than two) with a lower competition ratio, which would allow more target DNA to be applied to the gel than that used here. Besides DNA amplification and rearrangement, cloning of DNA sequences that become methylated or demethylated in a tissue-specific manner would also be possible by employing appropriate methylation-resistant (or sensitive) restriction enzymes. Although the procedure described above must be further improved for reproducible cloning sequences with a single copy or less per genome of mammalian origin, the current reassociation conditions may be already sufficient for cloning of low-copy sequences from lower eukaryotes such as yeast, which have less complex genome structures than mammals.

Several procedures are currently available to clone specific or altered DNA sequences from genomic DNA. Among them, Roninson (16) reported a procedure using in-gel reassociation of genomic DNA in the presence of excess amounts of driver DNA, which promote reassociation of amplified DNA sequences. The procedure has proved to be useful for selective cloning of amplified (or repetitive) DNA from mammalian genomic DNA. More recently, a method for detecting and cloning rearranged DNA sequences in a bacterial genome by using two-dimensional electrophoresis was reported (17). These procedures, which also use in-gel DNA reassociation, however, are different in principle from ours.

The isolation of apparently amplified or modified DNA sequences specific to brain from three groups with the cross-hybridizing DNA inserts analyzed may sound as if a significant number of similar brain-specific sequences are present in rats, considering the fact that we analyzed the clones from only a fraction (0.5–2 kb) of the original *Eco*RI-digested rat brain DNA. We feel, however, that the results must be carefully interpreted because DNA clones that give apparent tissue-specific unique Southern hybridization patterns could occur for various reasons. These include incomplete digestion of DNA, selective extraction of DNA from specific tissues, alteration in DNA structure in the particular rat used for the experiments without generality, or alteration caused by virus infection, as well as many others as discussed previously (15).

The uses of this procedure for the cloning of altered DNA sequences, particularly if perfected, are for the most part self-evident. In addition to tests for possible alterations in DNA primary structure during developmental processes, identification of sequences that allow distinction between individuals or species, as well as detection of chronological changes in DNA structure during tumorigenesis would be only a few of the possible applications.

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