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Mapping Replication Origins by Nascent DNA Strand Length

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The mapping of replication origins by nascent DNA strand length determination is a very sensitive generally applicable method that identifies even single-copy origins in mammalian chromosomes. A major advantage of this procedure is that there is no need for synchronization of cells or treatment with metabolic agents, which allows the origin to be studied under physiological conditions. This technique is based upon the amplification of specific sequence markers on nascent DNA strands that initiated replication within the region of the putative origin. Therefore, this method requires detailed sequence information of the locus to be analyzed. As a first step, nascent DNA of proliferating cells is pulse-labeled with BrdU followed by size fractionation and purification with anti-BrdU antibodies. The position of putative origins can then be determined via identification of the shortest nascent strands that can be amplified by PCR and hybridized to probes homologous to the amplified segments. Here, we give a detailed description of the theory behind the method and a full recipe for its application. Advantages and limitations of the procedure are discussed.

An intriguing and very elusive problem of the current biological research is still the identification of eukaryotic origins of DNA replication. There remain many unanswered questions concerning the initiation of DNA replication, while significant progress has been made in the understanding of proteins that are involved in replication fork progression or the pathways controlling the entry of a cell into S-phase.

So far only the origins of DNA replication in budding yeast *Saccharomyces cerevisiae* have been well characterized (reviewed in 1 and 2). Much less is known about mammalian replication origins (for review see 3). The characterization of these origins requires information on "replicators," i.e., *cis*-acting sequences essential for normal origin function (4), and on sequences that serve as "initiation sites" for the replicative machinery.

The most intensively studied origin of replication in higher eukaryotes is that of the dihydrofolate reductase (DHFR) gene of Chinese hamster ovary cells (5–7). As shown by several mapping methods, replication of this gene does not initiate randomly (8, 9). However, the size of the region in which an origin may be located is still a matter of dispute. Two-dimensional mapping techniques have provided evidence that initiation occurs at multiple sites spanning an approximately 55-kb region distal to the DHFR gene (10). In contrast, other methods, including determination of direction of fork movement (11), analysis of distribution of Okazaki fragments, determination of the bias in the template strand utilized for leading or lagging strand synthesis (12), and determination of the size and abundance of nascent strands across the locus (13) have delineated a relatively small region (0.45–3 kb) in which an origin may be located.

RATIONALE OF THE METHOD

The mapping of replication origins by nascent DNA strand length is a highly sensitive method first

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described by Vassilev and Johnson (14) that allows even the identification of single-copy origins in mammalian genomes. There is no need for metabolic inhibitors or synchronization, allowing the origin to be analyzed under physiological conditions.

While prokaryotic replication is relatively simple since bacterial and viral chromosomes duplicate as a single replicon, replication of eukaryotic chromosomes is much more complex and occurs at tens of thousands of different replicons throughout the entire genome of each cell nucleus. During replication in mammalian cells, each origin generates replication bubbles. In an asynchronous exponentially growing population these bubbles will vary from small ones that have just been initiated to large ones that are about to complete replication. To map an origin within a defined region, the shortest nascent DNA strands containing a unique DNA sequence of this region must be identified. An origin of bidirectional replication will be located at the center of these strands. If replication is unidirectional, the origin will be at one end.

The origin mapping approach by nascent DNA strand length is schematically presented in Fig. 1. This mapping method requires DNA sequence information since at least three unique DNA elements that are distributed within the region of the putative replication origin must be selected (Fig. 1, segments A to E). Unsynchronized cells are pulse-labeled with BrdU for a brief period of time. This generates a mixture of very short BrdU-labeled nascent DNA strands that were initiated during the pulse period and are located directly adjacent to the origin up to DNA strands of the length of replicons whose label is located extremely distal to the origin. This population of nascent DNA strands is separated from contaminating parental DNA according to chain length under denaturing conditions either by sedimentation through an alkaline sucrose gradient (14) or by electrophoresis in alkaline agarose gels (15). Newly replicated BrdU-labeled DNA in each fraction can be purified from fragments of unreplicated parental DNA by immunoprecipitation with anti-BrdU antibodies (16). Distinct parts within these DNA strands corresponding to the unique elements within the region of interest are amplified by PCR and hybridized to radiolabeled probes that are complementary to the unique DNA segments. The hybridization pattern is analyzed assuming that nascent strands become longer as replication forks move outward from origins, so that the probes that detect the shortest nascent strands should be located closest to origins.

DESCRIPTION OF THE METHOD

Choice of Unique DNA Segments and Primers

To identify a putative origin, unique DNA segments that cover the region of interest equally must be selected. A minimum of three target DNA segments is necessary. If there is enough sequence information at least five to six segments should be chosen. These target DNA segments should not contain repetitive sequence elements. To make the quantification of the hybridization signals easier later on, all probes should be of approximately the same length. The PCR primers flanking these sequence-specific targets should be of approximately 20 nucleotides in length and preferably have the same GC content to ensure identical PCR conditions. Furthermore, the primers should have a minimum of potential secondary structure, i.e., no palindromes, and they should not be complementary to each other, especially in the 3′ regions.

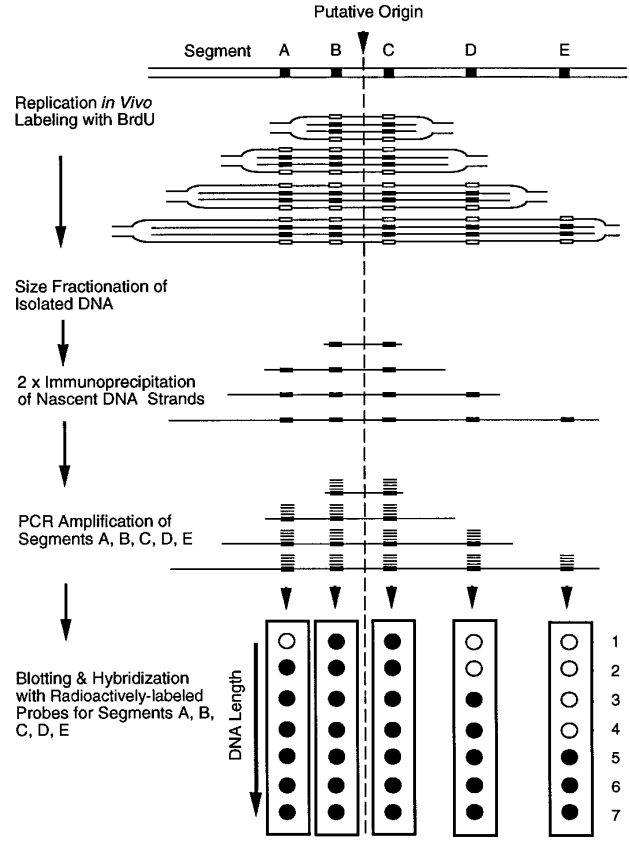

FIG. 1. Scheme of the origin mapping method by nascent DNA strand length.

Preliminary Testing of Primer and Probe Specificity

Preliminary PCRs with genomic DNA containing the putative origin region should be carried out to optimize the PCR conditions, e.g., hybridization temperature and magnesium concentration. The primers should generate a single product, and the size of the PCR products should be checked by gel electrophoresis to confirm their expected length. If the PCR primers do not generate a single band, but a major band and several minor ones, the major band could be excised from a gel and used in the assay. This allows some flexibility in the choice of PCR primers.

The specificity of the different probes should be checked by Southern blot hybridization (17). For each probe two blots are hybridized to the respective radiolabeled probe, one containing the unlabeled probe as a control and genomic DNA digested with an appropriate restriction enzyme, the second one containing the unlabeled probe as a control and the respective PCR product. All blots should exclusively generate one single band per lane. If the primers anneal specifically and amplify the correct segment, the signal of the PCR products should be at the same level as the control DNA.

Furthermore, cross-hybridization experiments should be carried out with the probes to ensure their specificity to each other. For each probe one dot blot is performed containing the respective unlabeled probe and each of the PCR products in concentrations varying from 0.1 to 5 ng. Filters are hybridized to the respective radiolabeled probes. They should generate signals only for the respective probe and the corresponding PCR product.

Labeling and Isolation of Nascent DNA Strands

As long single-stranded DNA strands are very fragile, the isolated DNA samples should never be stirred or vortexed during the purification and fractionation steps. This assay relies on the preparation of intact DNA strands. Once they are separated into distinct size classes, fragmentation is not a concern.

A total of 2×10^8 to 4×10^8 cells of semiconfluent cultures are incubated at 20 μM 5-bromo-2′-deoxyuridine (BrdU; Boehringer) for 15 min with constant agitation at 37°C. This period of time is sufficient to allow the synthesis of BrdU-labeled DNA regions of at least 20 kb. Since BrdU-labeled DNA is sensitive to UV-induced strand breaks, all of the following procedures should be done under minimal light. Wrapping the tubes with aluminum foil works well. Cells are collected by low-speed centrifugation for 2 min at 500*g*, washed three times with ice-cold phosphate-buffered saline, and lysed in 50 mM Tris–HCl, pH 8.0, 1 M NaCl, 10 mM EDTA, and 0.5% sodium dodecyl sulfate. Lysates are combined and incubated overnight with 100 μg of proteinase K (Boehringer) per milliliter. DNA is isolated by gentle inversion in phenol:chloroform (1:1) to avoid shearing. This step is repeated three to four times, and DNA is precipitated with 95% ethanol. DNA is gently dissolved in TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA).

Optional Incorporation of [^{3}H]Deoxycytidine

To monitor the efficiency of the labeling as well as the recovery during the isolation and purification procedure, cells may be labeled with 3 μCi [^{3}H]-deoxycytidine (19 Ci/mmol [^{3}H]dC; Amersham) in the presence of BrdU as described above. This step is not absolutely necessary but recommended, especially when establishing the method. The incorporation of [^{3}H]deoxycytidine into nascent DNA strands generates an additional internal control, e.g., when fractionating the DNA (see below). [^{3}H]Deoxycytidine incorporation increases proportionally with DNA length and the ratio of ^{3}H CPM to DNA length should be approximately constant for all gradient fractions, as would be expected for uniformly labeled size-fractionated DNA. During the immunoprecipitation step the loss of nascent DNA may be minimized, and 70 to 90% of the [^{3}H]BrdU-labeled DNA of each fraction should be recovered in the precipitate.

Fractionation of Nascent Strands According to Length

The fractionation of the nascent DNA strands must occur under denaturing conditions and can either be done by sedimentation through alkaline sucrose gradients or by electrophoresis in alkaline agarose gels. Both methods allow the recovery of DNA fractions ranging from e.g., 0.5 to 15 kb, although fractionation in alkaline agarose gels generates a better resolution of shorter nascent strands in the range of 0.5 to 3 kb and is more convenient in handling. Fractions containing nascent strands that are smaller than 500 bp should be discarded, since they represent a significant amount of not yet ligated Okazaki fragments, due to the semidiscontinuous mode of DNA synthesis. The same is valid for fractions larger than 15 kb, since these contain mainly unlabeled parental DNA.

Sucrose Gradient Centrifugation

The DNA is mixed with NaOH to a final concentration of 0.2 N (maximal final volume: 1 ml) and loaded onto a 5 to 20% (wt/vol) linear sucrose gradient (volume 32 ml) containing 0.2 N NaOH, 2 mM EDTA with a wide-bore plastic pipette. DNA is sedimented through the gradient for 18 h at 50,000*g* and 15°C in a swinging bucket rotor (e.g., SW28 rotor; Beckmann). Gradients are fractionated from the top to avoid contamination by viscous high-molecular-weight DNA at the bottom of the tube. Corresponding fractions from identical gradients can be combined. Each fraction is adjusted to 0.1 M Tris–HCl, pH 7.5, neutralized with 2 N HCl, and then supplemented with 100 μg of carrier DNA and ethanol-precipitated. Carrier DNA, e.g., salmon sperm DNA, must be purified by phenol extraction, degraded by boiling in 1 N NaOH for 1 h, and neutralized prior to addition.

The size distribution of DNA strands in each fraction of the alkaline sucrose gradient should be determined by alkaline agarose gel electrophoresis of an aliquot of each fraction against size markers.

Agarose Gel Electrophoresis

Isolated cell DNA samples are mixed with 1/6 vol of 6× gel loading buffer that consists of 300 mM NaOH, 6 mM EDTA, 18% Ficoll (type 400), 0.15% bromocresol green, 0.25% xylene cyanol and denatured for 3 min at 95°C. DNA samples are subjected to electrophoresis in an 1.2% alkaline agarose gel, approximately 15 cm in length, containing 50 mM NaOH and 1 mM EDTA. Adequate molecular weight markers are run in parallel (e.g., ranging from 500 bp to 14 kb; 1-kb DNA ladder (Gibco Brl) or bacteriophage λ *Hin*dIII DNA fragments). Gel electrophoresis is carried out in 50 mM NaOH, 1 mM EDTA at 0.7 V/cm for 16 h at room temperature. The alkaline agarose gel is stained in 0.5 M Tris–HCl, pH 7.8, and 0.5 μg ethidium bromide per milliliter. About 12 to 15 different fractions ranging from 0.5 up to approximately 15 kb are cut with a scalpel under UV light. Fractions of short strands should be taken in 0.3-kb intervals, e.g., ranging from 0.5 to 1.7 kb. The range may be increased to 1-kb intervals for strands of about 1.7 to 4.7 kb; for larger DNA strands fraction sizes will be 2 kb or more. Viscous high-molecular-weight DNA that is larger than 15 kb and stuck in the slots will be discarded. DNA from the different agarose fragments is eluted (e.g., in a Biotrap chamber, according to the manufacturer's instructions) and DNA ethanol precipitated in the presence of 100 μg carrier DNA (prepared as described above).

Immunoprecipitation of Nascent DNA Chains

DNA of each fraction is dissolved in 100 to 500 μl TE buffer, heat denatured for 3 min at 95°C in a 1.5-ml vial, rapidly cooled in an ice bath, and adjusted to 10 mM sodium phosphate, pH 7.0, 0.14 M NaCl, 0.05% Triton X-100 (immunoprecipitation buffer). Each fraction is incubated with 10 μl of mouse anti-BrdU monoclonal antibody (50 μg/0.5 ml; Boehringer) for 30 min at room temperature with constant shaking (16). Forty microliters of rabbit immunoglobulin (Ig) G directed against mouse IgG (2.8 mg/ml; Sigma) is added for 1 h at room temperature and constant agitation to precipitate BrdU–DNA–antibody complexes. Immunoprecipitates are collected by centrifugation at 400 rpm in an Eppendorf microfuge for 5 min, washed once with 0.5 ml of immunoprecipitation buffer, and then resuspended in 100 to 200 μl of 50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 0.5% sodium dodecyl sulfate, and 250 μg proteinase K per milliliter. At this step care must be taken not to lose the precipitate. DNA pellets are homogenized by vortexing, digested overnight at 37°C, and combined with 100 μg carrier DNA (prepared as described above). DNA is extracted once with an equal amount of phenol:chloroform (1:1), adjusted to 0.3 M sodium acetate, and then precipitated in ethanol. DNA is dissolved in 100 μl TE buffer and subjected to a second round of immunoprecipitation as described above, but this time, ethanol precipitation is carried out in the presence of 20 μg *Escherichia coli* tRNA (Sigma) instead of carrier DNA. The DNA precipitates are dissolved in 50 μl TE buffer and can be directly used for PCR. This second, independent round of immunoprecipitation in the presence of excess carrier DNA is required to reduce non-BrdU-substituted impurities to an insignificant level.

Alternatively purification of BrdU–DNA can be performed by immunoaffinity chromatography with anti-BrdU antibodies (18).

PCR Amplification

Polymerase chain reactions are performed for each size fraction, either by coamplification of the different target DNA sequences in one single tube upon addition of all primer pairs (only if there is no cross-reactivity between the different primer pairs) or by

amplification of one single target segment per tube. The latter leads to more variations, since many predictable and unpredictable variables might occur to one tube but not to the other. Such variations are, e.g., different PCR chambers or that in one single chamber the tubes are arranged near the edge and hence are slightly cooler than those in the center.

In parallel, a control amplification of the specific target segments, i.e., segments A to E, using 1 μg of unlabeled total cell DNA as a template, must be performed with the same PCR primers. This ensures that the absence of a target DNA in the subsequent blot hybridization is not due to inefficient amplification.

Polymerase chain reactions are carried out in a final volume of 100 μl containing picogram amounts of the DNA sequence of interest (e.g., 5 μl of each purified fraction), 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each deoxynucleotide, 1 μM each primer, and 3 units of *Taq* polymerase (Perkin–Elmer/Cetus). Amplification is carried out in a thermal cycler with the following cycle profile: an initial step at 93°C for 3 min, followed by 30 cycles at 93°C, 1 min (denaturation); primer annealing temperature (e.g., 56°C), 1 min (annealing); 72°C, 1 min (extension); and a final step at 72°C for 5 min. After 20 min at room temperature for complete annealing of the amplified strands, the DNA is twice extracted with phenol:chloroform (1:1) and ethanol-precipitated. Aliquots of the amplification products are analyzed in agarose gels containing 0.5 μg/ml ethidium bromide.

Blotting and Hybridization

PCR products and control amplified DNA segments are mixed with 0.1 vol 3 M NaOH, denatured at 68°C for 1 h, and quickly cooled in an ice bath. The samples are neutralized with 1 vol 2 M ammonium acetate, pH 7.0, and blotted to nylon membranes (Porablot; Macherey-Nagel) with a dot-blot device (Schleicher & Schuell). Membranes are washed twice with 0.5 ml 1 M ammonium acetate, pH 7.0, and baked for 2 h in vacuum at 80°C.

Filters are prehybridized with 0.1 ml/cm^2 of 0.5 M Na_2HPO_4, pH 7.2, 7% SDS for 2 to 4 h at 68°C (19). Radioactively labeled oligonucleotide probes (specific activity, 1×10^9 to 2×10^9 CPM/μg, labeled by random priming) are added and filters are incubated for 16 h at 68°C. Filters are washed with 0.4 M Na_2HPO_4, pH 7.2, 1% SDS for 5 min at room temperature, with 0.4 M Na_2HPO_4, pH 7.2, 1% SDS for 30 min at 60°C and with 0.1 M Na_2HPO_4, pH 7.2, 1% SDS for 60 min at 60°C. Filters are air-dried and analyzed by autoradiography and/or quantification using a PhosphorImager (Molecular Dynamics).

Analysis of the Hybridization Pattern

The shortest nascent DNA chains contain only the segments closest to the origin, whereas longer chains will contain two or more segments. Considering the hybridization pattern at the bottom of Fig. 1, the putative origin is located between segments B and C, which hybridize to the shortest nascent strands. This hypothetical origin is firing bidirectionally, since an unidirectional origin would generate an asymmetric hybridization pattern that would be displaced toward the position of either segment A or segment D. Measuring the minimum length of nascent DNA necessary to include a given segment allows the determination of the distance from that segment to the origin of replication. Therefore, to determine the position of the origin between segments A and E (for localization see Fig. 1 at the top), hybridization signals must be quantified. Quantitative comparison can be performed either by using a PhosphorImager and the Image Quant software or by scanning the autoradiographic patterns and determination of the optical densities from the surface of each peak. The resulting data are normalized to the average hybridization level of fractions that represent nascent DNA chains containing all five segments and therefore have B/A, B/C, B/D, B/E, C/A, C/B, C/D, and C/E ratios of 1. For example, normalization must be performed for all values of each blot hybridized to a specific probe, e.g., for fractions 1 to 7 of probe A. In Fig. 1 (bottom) fractions 5 to 7 contain nascent DNA strands that are long enough to generate uniform hybridization signals for all five probes, i.e., A through D. The mean of the values obtained for fractions 5 to 7 of probe A is calculated. This mean corresponds to 1. Subsequently, the values of fractions 1 to 4 must be normalized against the mean:

$$\text{mean (fractions 5 to 7)} \triangleq 1;$$

$$1 : \text{mean} = X$$

$$\text{value (of fraction 1)} : X = \text{normalized value (of fraction 1)}, \text{ etc.}$$

Now the ratios of ^{32}P-labeled probe that hybridized to B and C versus probes A, D, and E are calculated from their normalized values. These ratios will correct for variations that may occur between different

DNA segments during the amplification and hybridization steps. The hybridization ratios are then plotted as a function of DNA length. Figure 2 shows theoretical plots of hypothetical hybridization ratios for the pattern at the bottom of Fig. 1. The ratios of probe B versus ratios of probes A, C, D, and E are depicted. An analogous plot for ratios of probe C versus ratios of probes A, B, D, and E will be required.

Theoretically, if the nascent DNA chains in each fraction of the sucrose gradient or agarose gel are identical in length, then the ratio of B/A, B/D, and B/E and C/A, C/D, and C/E would change from infinity to 1 passing from a fraction containing chains shorter than the minimum length to a fraction containing chains longer than the minimum length necessary to hybridize to the appropriate probe. For each of the ratios B/A, B/C, B/D, B/E, C/A, C/B, C/D, and C/E two linear regression lines are drawn. The first line is drawn through points for which hybridization is high and nearly uniform for the five probes. This line should be nearly horizontal at a value of 1. The second line is drawn through points for which hybridization of probes A, D, and E is decreasing to nearly zero. These lines will differ for all probes. Each pair of lines forms a discontinuous curve. The projection of the inflection points (intersection of the two curves) of these curves to the abscissa gives the average size of the shortest hybridizing nascent DNA strands. Virtually the ascending portions of each discontinuous curve in the plots do not proceed directly to infinity, as might be expected. This is primarily due to the poor resolution of DNA in linear size gradients or alkaline agarose gels. Each fraction represents a distribution of sizes with an average peak value. The fractions overlap in size, and this contributes to the appearance of a slope in the regression curve instead of a sharp transition. Often, there are partial nascent DNA breakdown products present in the fractions.

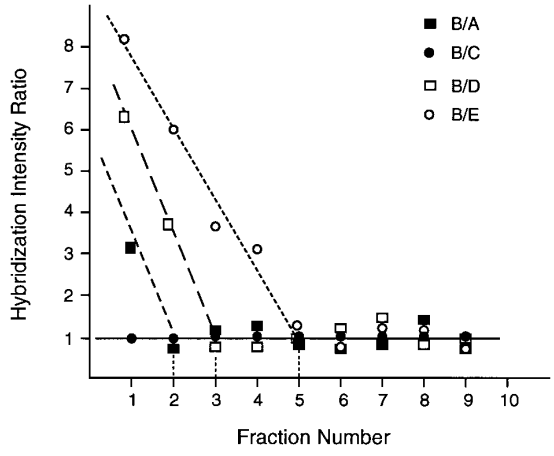

FIG. 2. Hypothetical hybridization intensity ratios of amplified DNA fractions from Fig. 1. Hybridization intensities of the dot blot (Fig. 1, bottom) are quantified. The ratios between B and A (solid squares) B/C (solid circles) B/D (open squares), and B/E (open circles) are calculated and plotted for each fraction. Two linear regression lines are drawn for each of the ratios. The first line is drawn through points for which hybridization is high and nearly uniform for the five probes. This line is nearly horizontal at a value of 1. The second line is drawn through points for which hybridization of probes A, D, and E is decreasing to nearly zero. Each pair of lines forms a discontinuous curve. The projection of the inflection points of these curves to the abscissa gives the average size of the shortest hybridizing nascent DNA strands.

Assuming that the newly replicated strands are a result of bidirectional chain growth from a centrally located origin, the average distances between the initiation site and the end of the amplification segments can be calculated as follows: The origin in Fig. 1 is located between segments B and C. Assuming that this origin is firing bidirectionally and that both hybridization signals are equal, the distance of B or C to this origin will be half of the distance between B and C. This position should be confirmed by the average length of the shortest nascent strands that hybridize to the other probes. In Fig. 2, the shortest hybridizing nascent strands are found in fraction 2 for probe A and in fractions 3 and 4 for probes D and E, respectively. These strands must have approximately twice the length estimated for the respective segment to the origin:

$$\text{distance B to C} : 2 \rightarrow \text{position of the origin}$$

$$\text{estimated distance of segment A to this origin} = Y$$

$$\text{average size of shortest hybridizing strands to probe A} = 2Y.$$

The degree of certainty attached to this calculation is enhanced by the number of fractions collected and by the number of segments amplified for hybridization. A potential error of approximately $\pm$ 500 bp (one size fraction) can be estimated for these calculations.

Interruption of the Procedure

The first possible stop is after the size fractionation of the nascent DNA strands, as from this step nascent strand breakdown is no longer a concern. Another possibility to interrupt the procedure is given after the immunoprecipitation step. In both cases samples should be stored at -20°C. The proto-

col might also be interrupted after the blotted samples have been fixed to the nylon membrane by baking for 2 h at 80°C in vacuum.

CONCLUDING REMARKS

Mapping replication origins by nascent DNA strand length is a very sensitive, relatively simple method to locate origins within a region of about 2.5 kb. One major advantage of this technique is that there is no need for metabolic inhibitors and cell synchronization, allowing origin function to be studied under physiological conditions avoiding possible artifacts. It is generally applicable to all species, since the efficiency of this method was first tested for the well-characterized SV40 origin of replication (14), although it would be the method of choice for any single-copy locus in randomly proliferating mammalian cells. This method allows the detection of uni- and bidirectionally firing origins of replication. If an origin has repeated sequences nearby, this method is still usable by simply selecting PCR primer sites outside of these repeats. Nevertheless, there are some limitations. Sequence information is required for the synthesis of appropriate PCR primers. This method is most suitable for mapping an origin in DNA regions < 10 kb in which the existence of an origin has been suggested previously by other data. It is too laborious to explore long genomic DNA regions. The DNA segments that are analyzed should not exceed a distance of 5 to 6 kb from the origin because of the light sensitivity of nascent BrdU-labeled DNA strands and the inaccuracy in separation of long DNA chains into distinct size fractions. To avoid artifactual signals, the PCR segments should not be located in proximity to repeated elements (e.g., *Alu* sequences), because of the increased rate of reannealing of nascent BrdU-labeled strands with unreplicated DNA of the same sequence during the immunoprecipitation step (20).

A modification of this method by Yoon *et al.* (21), the nascent strand abundance analysis, includes a two-step purification, based on the selection of short strands in an alkaline sucrose gradient followed by selection of fully BrdU-substituted strands by isopycnic centrifugation. With this modification there is no need for the PCR step. The nascent strand abundance analysis has the advantage of relative insensitivity to possible nascent strand breakdown during the later stages of nascent strand purification. This method works well in conjunction with the nascent strand size analysis, generating more quantitative information since it can distinguish between sites that fire more frequently than others (see also Huberman, this issue (21a)).

Application of another modification of the method by Vassilev and Johnson (14), the origin mapping by competitive PCR (22) generates a more precise restriction of the origin region down to about 500 nucleotides. This method entails the absolute quantification of the abundance of selected DNA fragments along a genomic region within samples of newly synthesized DNA by competitive PCR. The latter is immune to all uncontrollable variables which could severely affect the reproducibility of conventional PCR (see also Giacca *et al.*, this issue (22a)).

The mapping approach for replication origins by nascent DNA strand length has localized several origins of DNA replication: An origin of bidirectional replication within a 2-kb initiation zone in single-copy sequences of the c-myc gene locus in human cells (20); within a 2.5-kb initiation zone, centered about 17 kb downstream of the DHFR gene in CHO cells (13); and an origin of bidirectional replication within an 1- to 2-kb region of the ADA gene locus in mouse cells (23). Further origins of bidirectional replication have been mapped in human embryonic lung fibroblasts (24), the human heat shock 70 gene promoter (25), within a 1.6- to 2-kb region in the CHO *RPS 14* gene (26), and to a single-copy chromosomal locus in *Drosophila* (27). Other origins of bidirectional replication are located within a 1-kb initiation zone containing the aldolase B promoter (28), in the mouse ribosomal gene cluster within a 3-kb region centered about 1.6 kb upstream of the rDNA transcription start site (15), and in rat and human rRNA genes (29).

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