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Mapping initiation sites of DNA replication *in vivo* using polymerase chain reaction amplification of nascent strand segments

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ABSTRACT

We describe a sensitive method for mapping replication initiation sites near regions of sequenced genomic DNA *in vivo*. It is based on selective amplification of sets of segments in purified nascent DNA strands and subsequent determination of the lengths of these strands required to include each member of the set. We demonstrate the ability of this method to accurately map a well-defined origin, that of replicating SV40 DNA. Pulse-labeled DNA from infected CV-1 cells was size-fractionated on an alkaline sucrose gradient and newly-synthesized strands purified by immunoprecipitation using anti-BrdU antibodies. Three pairs of synthetic oligonucleotide primers were used to amplify three SV40 segments, using the polymerase chain reaction (PCR), at known distances from the origin. Lengths of the nascent DNA strands that allow amplification were determined by hybridization to probes homologous to the amplified segments and used to calculate position of the origin. Experiments with a mix of SV40 and human HeLa cell DNA demonstrate the applicability of the method to mapping origins present at the level of single-copy genomic sequences in mammalian cells.

INTRODUCTION

Advances in understanding the control of DNA replication in mammalian cells now require that we be able to locate initiation sites at the level of nucleotide sequences in replicating genomic DNA. Replication of DNA in eukaryotic chromosomes initiates at multiple sites, termed origins, spaced at intervals along the genome (1,2). Autoradiographic and BrdU pulse-labeling experiments have shown that the vast majority of origins initiate bidirectional replication (2). Despite much progress in understanding the enzymology of replication, many questions regarding the specificity of initiation remain to be answered (cf 3). In lower eucaryotes instances of distinct sequence specificity for initiation have been noted. Specific origins of replication have been mapped on the linear rDNA minichromosomes of *Physarum* (4,5) and *Tetrahymena* (6). Specific sequences (ARS) have been shown to confer upon plasmids the ability to replicate autonomously in yeast, and these have been hypothesized as chromosomal origins (reviewed in 7). Recent mapping experiments suggest that at least a fraction of the ARS sequences serve to initiate replication in the yeast 2μ plasmid (8,9), in yeast chromosome III (10) and in yeast ribosomal gene repeats (11). Localizing origins in higher eukaryotes has been much more difficult. Differential gene amplification is believed to involve origins located within distinct chromosome regions in a variety of systems (12−14). Recent reports based on polarity of fork progression suggest that origins may be present in the vicinity of avian alpha globin (15), human c-myc (16) and human APRT (17) genes, although locations of possible initiation points have yet to be identified. Several isolation procedures have yielded cloned mammalian DNA sequences reported to confer

ARS activity on plasmids (18−21), but at this point no reliable mapping procedure exists to test potential chromosomal function of any of these.

Chromosomal mapping procedures for replication origins published so far are of limited applicability to mammalian systems. Methods developed to ascertain polarity of DNA replication through a given sequence (15−17) do not provide sufficient resolution for precisely locating initiation sites. Other methods employ hybridization of probes to restriction fragments containing genomic DNA replication intermediates separated by 2-dimensional gel electrophoresis (8,22). These methods have been useful in determining that ARS sequences serve as initiation sites in certain yeast locations (8−10). The necessity to detect replicating intermediates as gel configurations, however, drastically limits the sensitivity of such methods. The vast numbers of proliferating cells required renders these methods impractical for most studies of origins present at the level of a single-copy genomic sequence in mammalian cells (22). The mapping procedures described are thus far limited either to lower eukaryotes, with a genomic complexity approximately 1/100 that of humans, or to highly repetitive systems where the hybridization signal from a single origin may be enhanced.

We have applied two recent technological advances to develop a highly sensitive method for mapping newly-initiated DNA chains. Amplification of specific DNA segments via a DNA polymerase chain reaction (PCR) has proven effective for detection of single-gene-copy sequences isolated from minute quantities of cells (23,24). This method allows amplification of segments of isolated DNA single strands. Improved ability to purify nascent, newly-synthesized DNA strands from parental strands after BrdU incorporation has recently been reported by Vassilev and Russev (25). Combining these advances, we describe here a method for amplifying segments of newly-synthesized strands containing known genomic sequences. Using sets of probes spaced at discrete intervals, we can map the initiation points of these nascent strands. We have employed this method with a test system, SV40 DNA replicating in monkey CV-1 cells. The SV40 replication origin has been well-characterized (26) and initiation sites mapped (27), and it has been used as a standard model system for previous origin isolation (28) and mapping (22) techniques. We document here the sensitivity of this method and its applicability to mapping initiation sites near single-copy genomic DNA sequences in mammalian DNA.

MATERIALS AND METHODS

Labeling of DNA in cultured cells.

African green monkey kidney cells were grown in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal calf serum under 5% CO_2. At near confluency cells were infected with SV40 (20 PFU units per cell). They were labeled 32 hrs later for 5 min with ^{3}H-deoxycytidine (5 μCi/ml, 25 Ci/mmol, New England Nuclear) in the presence of 50 μM 5-bromodeoxyuridine (BrdU, Boehringer). Cells were washed with cold phosphate buffered saline (PBS) and lysed in 0.6% SDS, 10 mM EDTA, pH 8.0. NaCl was added to a final concentration of 1M and after 10 min in the cold cell lysates were centrifuged for 20 min at 11,000×g. This modification of the Hirt procedure (29) isolates most of the viral DNA together with a significant amount of cellular DNA. Suspension cultures of HeLa cells were maintained in spinner flasks with DMEM and 10% fetal calf serum. Aliquots of HeLa cell suspension were labeled 5 min with ^{3}H-deoxycytidine (10 μC/ml) in the presence of 50 μM BrdU with constant agitation at 37°C. Cells were collected by low speed centrifugation, washed with PBS and lysed in 0.5%

SDS, 1 M NaCl, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0. Proteinase K (Boehringer) was added to make 0.2 mg/ml, and samples were incubated for 2 hrs at 37°C. DNA was extracted with phenol and phenol-chloroform (1:1) and precipitated with ethanol.

Gradient centrifugation and length measurements of single-stranded SV40 DNA fragments.

An aliqot of the supernatant CV-1 DNA containing approximately 15 μg SV40 DNA was made 0.2 N in NaOH and loaded on top of a 5−20% linear sucrose gradient containing 0.2 N NaOH. It was run for 16 hrs at 50,000×g at 20°C in a Beckman 50.1 rotor. The gradient was unloaded from the bottom by means of a peristaltic pump and fractionated into 0.45 ml fractions. Aliquots (50 μl) were taken from the alkaline gradient fractions and assayed for radioactivity. Each alkaline gradient fraction was neutralized with HCl in the presence of 0.1 M Tris-HCl buffer, pH 7.5, and DNA collected by ethanol precipitation. DNA aliquots from each gradient fraction were alkali-denatured and subjected to electrophoresis in an alkaline 1% agarose gel (30). SV40 DNA restriction fragments (a mixture of Bam H1, Hind III and Pst I digests) were run in parallel to serve as molecular weight markers. Gels were Southern blotted on Gene Screen Plus membranes and hybridized with nick-translated SV40 DNA and the size distribution of SV40 DNA fragments determined for each gradient fraction.

Immunoprecipitation of nascent DNA chains.

BrdU-containing nascent DNA strands were purified from bulk DNA by immunoprecipitation using slight modifications of an earlier procedure (24). Briefly, 200 μg of HeLa DNA (labeled *in vivo* for 10 min with ^{3}H-deoxycytidine and BrdU and sonicated to 1−5 kb) were mixed with aliquots of each alkaline gradient fraction (containing nanogram amounts of SV40 sequences). The mixture was denatured for 2 min at 95°C. After rapid cooling a monoclonal anti-BrdU antibody (Becton-Dickinson) was added in a final volume of 0.4 ml, and samples were incubated for 20 min at room temperature with slow agitation. The antibodies were in a slight molar excess over the calculated amounts of BrdU-DNA. After addition of the second antibody (rabbit anti-mouse IgG fraction, Sigma) and another 20 min incubation, precipitates formed were pelleted by centrifugation for 5 min in an Eppendorf microfuge. They were washed once with PBS and resuspended in 0.2 ml of 0.5% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0. DNA was deproteinized by digestion overnight with Proteinase K (0.2 mg/ml) followed by two phenol-chloroform and three ethyl ether extractions. The amount of DNA precipitated was estimated by UV absorbance at 260 nm. To each fraction tRNA (5 μg) was added, and DNA was precipitated with ethanol, dried and dissolved in 50 μl of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

PCR amplification.

Two DNA primers 20 nucleotide long were selected for each amplification from the sequence of SV40 DNA (see Fig 2). They each contained 9 GC pairs and no obviously complementary regions. In addition three more 21-mer oligodeoxynucleotides were selected from 3′−5′ strands between each of the amplification primers. The exact positions of all 6 oligonucleotides on SV40 DNA are shown in Fig 2. They were chemically synthesized by the phosphoramidite method using an Applied Biosystems 380 B DNA synthesizer and purified from the incomplete synthetic products by means of OPC cartridges (Applied Biosystems) as recommended by manufacturer. Polymerase chain reactions were performed in a final volume of 100 μl containing: DNA sequence of interest (picogram amounts); 10 mM Tris-HCl,pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% gelatin; 0.2 mM each of dATP, dGTP, dCTP and TTP; 1 μM of each oligodeoxynucleotides primer and 3 units of Taq Polymerase (Perkin Elmer Cetus). Amplification was carried out in the Perkin Elmer

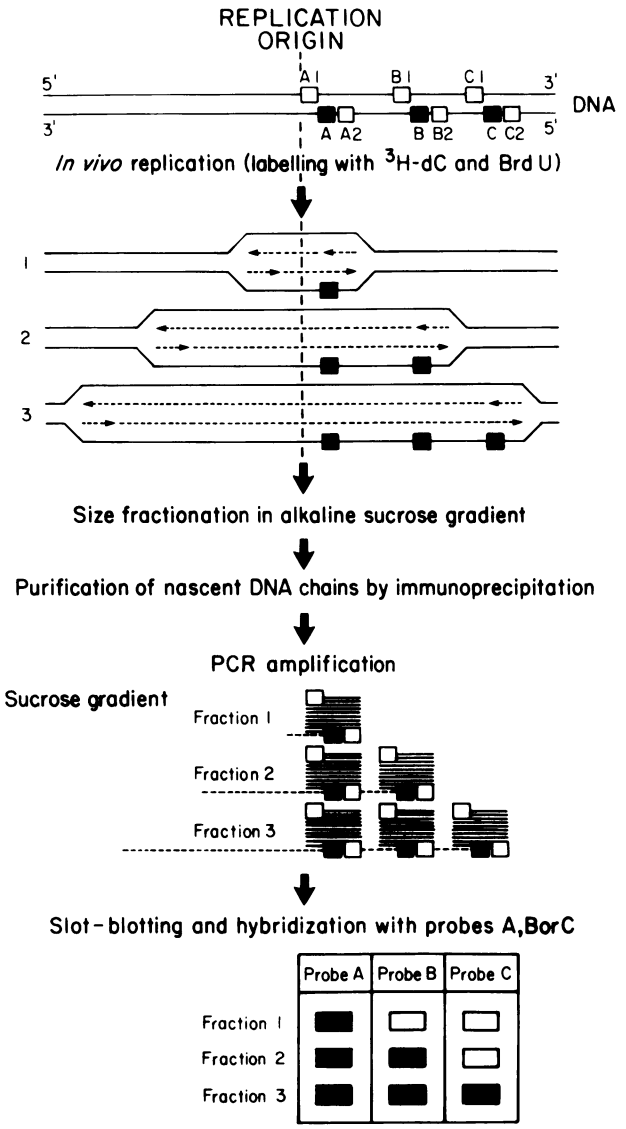

Fig 1. *Mapping of replication origins by PCR amplification of segments on nascent DNA strands.* Newly replicated DNA strands are presented with dotted lines. Arrows indicate the direction of DNA synthesis. Empty boxes—PCR amplification primers; black boxes—hybridization probes.

Cetus DNA Thermal Cycler with a cycle profile as follows: denaturation for 1 min at 94°, annealing for 2 min at 53°, extension for 1 min at 70°. Rate of temperature transition between the steps was 1 min. 30 cycles were used for each amplification reaction. After 20 min at room temperature for complete annealing of the amplified strands, DNA was extracted twice with phenol-chloroform (1:1) and precipitated with ethanol. Aliquots of

the amplification product were analysed in 2.5% agarose gels containing 0.5 μg/ml ethidium bromide.

Blotting and hybridization.

DNA was blotted onto Gene Screen Plus membranes (New England Nuclear) using a Schleicher & Schuell Minifold II slot-blot system. Total SV40 DNA (Biolabs) was labeled with α-^{32}P dCTP (3000 Ci/mmol, NEN) by nick-translation. Oligonucleotide probes were 5-end labeled with T4 polynucleotide kinase and γ-^{32}P ATP (3000 Ci/mmol, NEN). Labeled DNA or oligonucleotides were purified from unincorporated label by a passage through Sephadex G50 or G25 columns, respectively (Quick Spin columns, Boehringer). For hybridization with nick-translated DNA probes membranes were preincubated 2 hrs in 1 M NaCl, 50 mM Tris-HCl,pH 7.5, 1% SDS and 0.1 mg/ml denatured salmon sperm DNA. They were hybridized overnight in the same buffer at 65° with labeled probe (10^6 cpm/ml) and washed twice for 1 hr with at 65° with 2×SSC,1% SDS. For hybridization with the oligonucleotide probes, membranes were incubated 2 hrs at 55° in the same prehybridization buffer as above but with 0.1 mg/ml carrier salmon DNA hydrolysed to oligonucleotides by alkali treatment. Hybridization was in the same solution for 2 hrs at 55° (10 degrees less than their dissociation temperature) with 10^6 cpm/ml (specific radioactivity of the probe 2×10^8 cpm/μg). They were washed twice for 15 min at room temperature and for 1 hr at 58° with 2×SSC,1% SDS. Autoradiography was for 3 hrs at room temperature using Kodak X-OMAT film. Autoradiographs were scanned with a Bio Rad Model 620 Video Densitometer, and the optical density of the spots calculated from the peak areas.

RESULTS

Mapping start sites of nascent DNA strands.

The origin mapping approach we used is schematically presented in Fig.1. For any origin of replication situated near a genomic DNA segment of known sequence, we can synthesize three pairs of oligonucleotides with members complementary to both DNA strands downstream of the origin (A1,A2; B1,B2; and C1,C2 in Fig 1). These will serve as primers for specific amplification of the regions between each pair by the polymerase chain reaction. An additional set of oligonucleotides (A, B and C) are synthesized as specific probes for each amplified DNA segment. In unsynchronized cells the initiation of DNA synthesis at the origin and subsequent elongation produce a population of newly replicated DNA strands ranging in size from 0 to full chromosome length. If the cells are labeled shortly with ^{3}H-deoxycytidine in the presence of BrdU and the isolated DNA run in an alkaline sucrose gradient, we can physically separate the population of relatively short nascent BrdU-DNA from the bulk DNA and fractionate it into different size classes. Newly replicated BrdU-DNA can be efficiently purified from unreplicated DNA in each fraction by immunoprecipitation with anti-BrdU antibodies (25). The sequences to be probed in each purified nascent DNA fraction are then amplified 10^5-10^6-fold by PCR in the presence of all three pairs of primers. The high concentration of primers used prevents any amplification of overlapping segments since the Taq polymerase used does not displace the primers. Thus, depending on their initial size, nascent DNA strands will allow amplification of either segment A, segments A and B, or segments A,B and C. This can be detected by hybridization of the blotted DNA fractions with probes A, B or C. From the diagram in Fig 1 is clear that the pattern of amplification and hybridization of strand size fractions to the various probes will show the direction of fork progression through

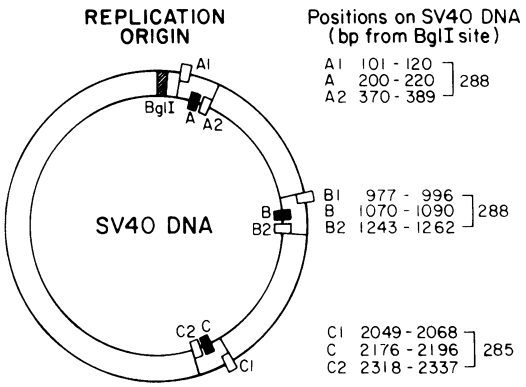

Fig 2. *Positions of PCR-amplified segments on SV40 DNA.* Shaded box—replication origin region; empty boxes—PCR amplification primers; black boxes—hybridization probes.

the sequences. Furthermore, the size of the shortest DNA fraction which allows amplification of a sequence will determine the distance between the origin of replication and the end of that sequence. Locating the initiation site by this method is most straightforward for origins of bidirectional replication with relatively symmetrical fork progression. In that case the origin is mapped to a point half the length of the shortest strand hybridizing to each specific probe. Details of the mapping calculations are further considered in the discussion.

We tested the ability of the method described to detect a known origin active in cultured

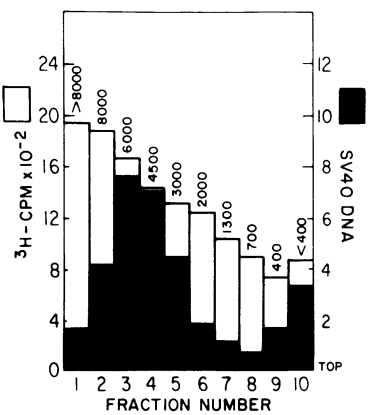

Fig 3. *Separation of pulse-labeled DNA strands by alkaline sucrose gradient centrifugation.* Virus-enriched cellular DNA was isolated from SV40-infected CV-1 cells pulse-labeled for 5 min with ^{3}H-deoxycytidine and BrdU. An aliquot was alkali denatured and single-stranded DNA separated on a 5–20% alkaline sucrose gradient as described in Materials and Methods. Radioactivity of each fraction was determined by scintillation spectrometry of aliquots. Separate aliquots were used to determine the amount of SV40 sequences by slot-blotting and hybridization with ^{32}P-labeled SV40 DNA. Size distribution of DNA fragments in each fraction was determined by analytical alkaline agarose gel electrophoresis as described in the text. Size distribution was as follows (in nucleotides): fraction 1: >8000; fr.2:5000–10000; fr.3:4000–8000; fr.4:3000–6000; fr.5:2000–4500; fr.6:800–3000; fr.7:600–2000; fr.8:300–1000; fr.9:200–700; and fr.10: <400. □—3H-radioactivity, ■—relative amount of SV40 DNA in arbitrary units. Numbers indicate the average fragment size (mid peak value in nucleotides) for each gradient fraction.

mammalian cells, i.e. SV40 virus replicating in CV-1 cells. We chose three amplification segments (A, B and C; approximately 0.3, 1 and 2 kb downstream from SV40 replication origin, respectively) and corresponding oligonucleotide primers (A1,A2,B1,B2,C1,C2; all 20-mers) were chemically synthesized (Fig 2). Three other 21-mer oligonucleotides were also synthesized to serve as specific hybridization probes for each amplified segment. Due to the semidiscontinuous mode of DNA synthesis, a significant amount of not-yet-ligated Okazaki fragments, released upon denaturation, would accumulate in the low molecular weight fractions of the alkaline sucrose gradient. These short fragments would carry virtually all the sequences of SV40 DNA and would be expected to minimize or completely abolish the expected difference in the hybridization pattern. In order to avoid this, we chose the lengths of the amplification segments to be 280−290 nucleotides, a value greater than the average length of Okazaki fragments, 114 bp, reported for replicating SV40 (31). Thus individual Okazaki fragments would not be amplified, even in fractions where they are abundant, since both primers could hybridize simultaneously to only a small minority of them.

Isolation and fractionation of nascent SV40 DNA.

Simian virus 40 infected CV-1 cells were incubated for 5 minutes with 3H-deoxycytidine and BrdU to label newly replicated DNA. Replication was stopped by brief washing and immediate lysis of the cells and DNA was isolated as described. An aliqot of the CV-1 DNA (containing approximately 15 μg of SV40 DNA) was alkali denatured and run in a linear 5−20% alkaline sucrose gradient. Centrifugation conditions were prestandardized to allow fractionation of DNA strands in the range of 500−10000 nucleotides. Fig.3 shows the ^{3}H profile of the gradient and the estimated sizes of the single stranded DNA in each fraction. Under the conditions used, replicated, fully-closed circular SV40 DNA molecules equilibrate at the very bottom of the gradient. The linear SV40 strands are present in the gradient with denatured CV-1 genomic DNA strands, which comprise the bulk of labeled material. When aliqots of the gradient fractions are slot-blotted and hybridized with a nick-translated SV40 DNA, the amount of SV40 sequences in each fraction corresponds well to that expected for each size class. As expected, an accumulation of SV40 sequences in the top two fractions, most likely representing Okazaki fragments, is also observed.

Immunoprecipitation of nascent DNA strands.

Monoclonal antibodies against BrdU are effective for preparative purification of newly replicated BrdU-DNA strands (24). This technique is faster and simpler and the resolution better than that obtained with conventional equilibrium centrifugation. The application of the immunoprecipitation procedure, however, is limited to a certain extent by the fact that if highly repeated sequences are present in the BrdU-DNA to be purified, some annealing could occur during antigen-antibody reaction. This could cause indiscriminate trapping of non-replicating repeats with precipitated nascent DNA. On the other hand, C_0t conditions are highly unfavorable for annealing of lowcopy or unique sequences from eukaryotic cells subjected to the immunoprecipitation procedure (L. Vassilev and E.M. Johnson, unpublished observations). To avoid possible effects of such annealing on purification of nascent SV40 DNA, as well as to test the sensitivity of the mapping method, we mixed aliqots from gradient fractions 1 through 9 (Fig 3), containing from about 0.3ng (for the shortest) to about 3ng (for the longest fractions) with 200 μg of total HeLa cell DNA labeled for 10 min with ^{3}H-deoxycytidine and BrdU. This dilution of SV40 DNA (approximately 6×10^5-fold) made it as concentrated as a single-copy DNA sequence 5 kb long in the human genome. Each fraction was then immunoprecipitated with anti-BrdU

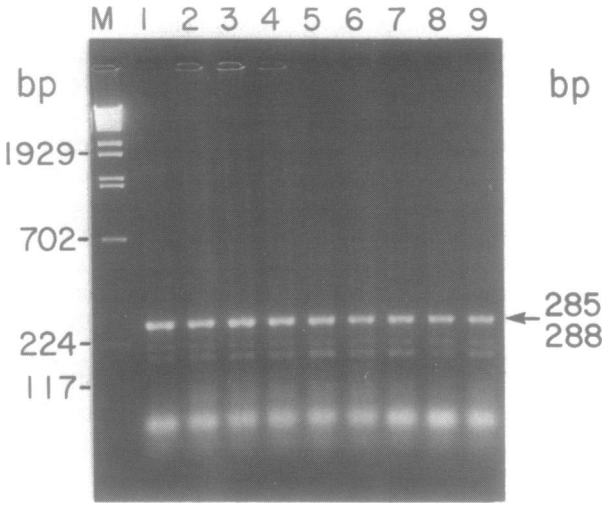

Fig 4. *Agarose gel electrophoresis of the PCR-amplified, nascent SV40 DNA fractions.* Immunoprecipitated nascent DNA chains from fractions 1 through 9 of the sucrose gradient were subjected to 30 cycles of amplification in the presence of all three pairs of specific SV40 PCR primers (see Fig 2). Aliquots of the amplification products were subjected to electrophoresis on a 2.5% agarose gel containing 0.5μg/ml ethidium bromide. Lines at left indicate the positions of selected marker fragments of lambda phage DNA digested with BstEII. Arrow at right indicates the position of the 285−288 nucleotide amplification products.

antibodies and the ^{3}H radioactivity counted in aliquots of purified DNA. In agreement with our previous data, between 70 and 88% of the labeled DNA from each fraction precipitated together with less than 1% of the optical density, which corresponded well to the expected value for DNA synthesized during a 10 min pulse in unsynchronized population of HeLa cells (25).

Specific amplification of nascent SV40 DNA strands.

The recently developed PCR amplification technique is based on the ability of DNA polymerases to extend 3′-ends of a pair of oligonucleotide primers complimentary to both DNA strands and by many repeating cycles to produce multiple copies of the sequence closed between the primers (32). A specific $10^{5}-10^{6}$-fold amplification of single copy sequences make them easily detectable even in the most complex mammalian genomes (23). Here we used this technique to amplify short segments of the nascent DNA strands synthesized bidirectionally from a replication origin. Amplification $>10^{5}$-fold permits detection of femtogram amounts of a specific DNA sequence. Control amplification reactions were performed using different SV40 synthetic primer pairs with 10 pg of total SV40 DNA. After 30 PCR cycles aliquots of the reaction mix were analyzed on an agarose gel. Single bands with the expected size of about 280 bp were clearly visible on a faint smeared background with multiple weak bands (not shown). An amplification of $0.5-1.0\times10^{6}$ was estimated by comparison with ethidium-stained standards.

One fifth of DNA in each immunoprecipitated DNA fraction (estimated DNA content of approximately 1 pg for the shortest fractions) were amplified under the same conditions as the above controls in the presence of all three pairs of oligonucleotides. The electrophoretic pattern of the amplification products in all 9 fractions (Fig 4) showed a predominant band approximately 280 bp long. The intensities of amplified DNA segments

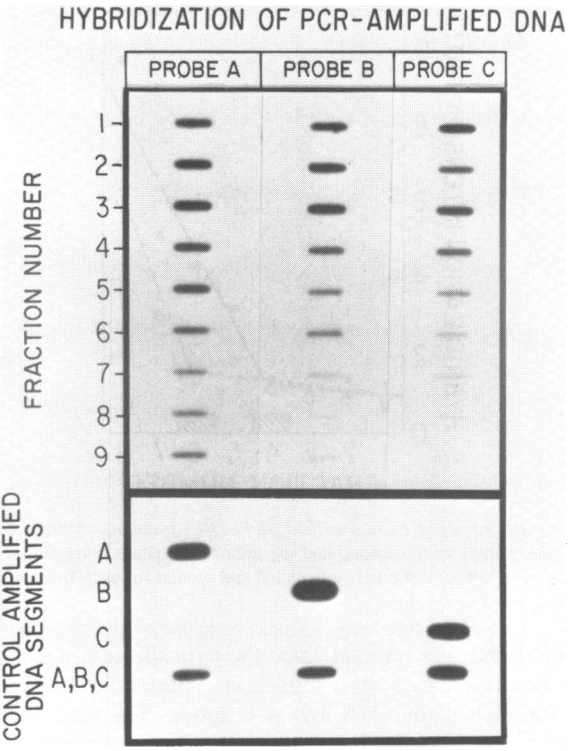

Fig 5. *Slot-blot hybridization of PCR-amplified SV40 DNA.* Upper panel: aliquots of each DNA size fraction were taken after PCR amplification (see Fig 4), slot-blotted in three identical patterns and hybridized with ^{32}P-end-labeled oligonucleotide probes A, B or C (see Fig 2). Lower panel: aliquots of control reactions performed with a mixture of 10 pg of SV40 DNA and 100 ng of HeLa DNA either in the presence of each oligonucleotide primer pair A, B or C separately, or in the presence of all three primer pairs together, were blotted and hybridized as above.

in the different size fractions were nearly equal, indicating that at least one of the amplified sequences is present in even the shortest of the nascent chains. As a specificity control another four amplification reactions were performed under exactly the same conditions as above but with a mixture of 10 pg of SV40 DNA and 100 ng of total HeLa DNA with each oligonucleotide pair A, B or C and all together, respectively. One fifth of the PCR amplificated DNA samples were slot-blotted on nylon membranes in three identical rows and hybridized with the end-labeled oligonucleotide probes A, B or C (Fig 5). This control demonstrates that the PCR amplification, coupled with oligonucleoitide hybridization, results in a high degree of specificity with practically no background hybridization. It can be seen in Fig 5 that oligonucleotide probes A, B and C show differences in their pattern of hybridization with the PCR-amplified nascent DNA size fractions. Probe A hybridized with all fractions. Probes B and C had almost the same intensity of hybridization as probe A for fractions 1 through 4 and a significant relative decrease after fraction 6 and fraction 4, respectively. For quantitative comparison autoradiographic patterns were scanned and optical densities determined from the surface of each peak. The density ratios for each fraction were calculated as A/B and A/C and graphically presented in Fig 6. Fraction 9

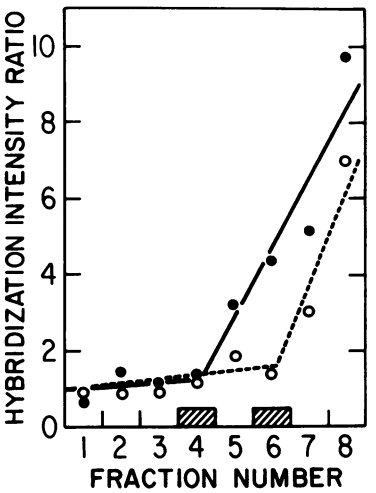

Fig 6. *Hybridization intensity ratios of PCR-amplified SV40 DNA fractions.* Autoradiographs of the slot-blot hybridization (Fig 5, upper panel) were scanned and the area of the peaks determined. The ratios between A and B (○- - -○) and A and C (●——●) were calculated and plotted for each fraction for fractions 1 through 8 of the sucrose gradient (see Fig 3).

(at the top of the gradient) was omitted since the hybridization intensity ratio for it was still influenced by the presence of short replication intermediates. Two linear regression lines were drawn for each of the A/B and A/C ratios. The first line is drawn through points for which hybridization is high and nearly uniform for the three probes. This line is nearly horizontal at a value of 1. The second line is drawn through points for which hybridization of probes B and C is decreasing to nearly zero. These lines differ for the two probes. Each pair of lines forms a discontinuous curve. The curves thus drawn generally reflect the theoretical model although certain factors may contribute to their more complex character, as considered in the discussion. The projection of the inflection points of these curves to the abscissa gives the average size of the shortest hybridizing nascent DNA strands. In our mapping experiment this was fraction 6 for probe B and fraction 4 for probe C, with average sizes of 2000 nucleotides and 4500 nucleotides, respectively. Accepting that that newly replicated SV40 strands are a result of bidirectional chain growth from a centrally

TABLE 1. Summary of SV40 DNA origin mapping data

PCR-amplified sequence	Shortest origin containing fragment carrying A, B or C[a] (n)	Estimated distances between A, B or C and replication origin (n)	Actual distance from Ori (BglI site)
A	400	n.a.[b]	389
B	2000	1000	1262
C	4500	2250	2337

[a] From Fig 6 and Fig 3.
[b] Not applicable since probe A hybridized to all fractions indicating close proximity to a potential origin.

located origin (33), we calculated the average distances between the initiation site and the end of the amplification segments (Table I). A potential error of approximately ±500 bp can be estimated for these calculations based on size distribution in individual fractions. The degree of certainty attached to this calculation is enhanced by the number of gradient fractions collected and by the number of segments amplified for hybridization. It can be seen in the last column of Table I that the experimentally-determined position of the replication initiation site, based on the size averages of gradient fractions and obtained using two amplified segments, corresponds closely to the known position of the SV40 origin.

DISCUSSION

One of the primary advantages of the mapping method described here is its sensitivity. In a nonsynchronous population of mammalian cells, at any given time the amount of newlyreplicated, nascent DNA encompassing a specific sequence of interest is very low. Thus in studying replication of a sequence present at a single genomic copy, one must contend not only with low copy number, but also with the low level of pulsed, nascent DNA per cell. For mapping methods previously described, this necessitates working with vast numbers of proliferating cells. In theory the amplification achieved by PCR can be virtually unlimited. We employed 30 replication cycles, resulting in nearly 10^6-fold amplification. Fig 4 shows that this level of enhancement allows visualization of agarose gel bands of amplified 280-nucleotide segments. Since the specificity of hybridization to the amplified segments is background-free (Fig 5), even higher levels of amplification are feasible. The PCR amplification approach was developed mainly to help solve the problem of sensitivity in mapping mammalian origins. However, since no mammalian origin has yet been unequivocally located with any degree of accuracy, we used instead a standard SV40 DNA system as a model. As prepared and mixed by us with HeLa cell DNA, the SV40 sequence resembles a 5 kb single-copy segment replicating in the human genome. The only significant difference lies in the different lengths of the viral and cellular replication cycles. With approximately 15−30 min per viral cycle, SV40 generates many more short nascent DNA strands than the same amount of single copy DNA in cells with average 20 hour cell cycle. Under the experimental conditions used here a single round of purification by immunoprecipitation gave satisfactory resolution for the slot-blot hybridization analysis. Additional purification may be applied, however, in experiments with mammalian cells. A second round of immunoprecipitation and/or equilibrium centrifugation in CsCl has proven successful for purification of BrdU-DNA from a great excess of contaminating total DNA (L.Vassilev, unpublished results). In the present experiments we were able to map the replication origin in few nanograms of SV40 DNA using less than 10% of the final material from each sucrose gradient fraction. We estimate that with the same amount of total DNA and with only 5 more amplification cycles, the level of SV40 DNA that would allow mapping can be lowered to as little as few picograms. This means that it is technically feasible to map a single-copy origin in as little as 100 μg of pulse-labeled human cellular DNA, the amount extracted from about 10^7 cells. A current 2-dimensional gel mapping method reportedly requires approximately 200-fold higher levels of human cellular DNA (22).

Clearly two elective factors contribute to the accuracy of the method as presented in Figs 5 and 6. One is the size of each fraction taken from the alkaline sucrose gradient. The other is the number of amplified segments used. For the trial study shown here, we collected gradient fractions that limit our accuracy to about ±500 bp. As seen in Fig 6,

this limit was met by using only two amplified segments. In practice with genomic DNA, use of three to five amplified segments, each located >0.5 kb from the origin, should suffice to yield statistically accurate mapping. Best results should then be achieved by amplifying from separated nascent strands ranging from 0.5 to 10 kb in size.

In calculating the position of an origin from data obtained, it is possible that some error could be introduced by the lag in hybridization of the last Okazaki fragment(s), which would affect one end of each nascent strand sized on the alkaline sucrose gradient. Clearly the percent error would be greater for probes located very near the origin. Data presented here show that, at least for one system, SV40 in CV-1 cells, the effect of this lag on localization of origins several kb from each given sequence is minimal.

Initial mapping calculations for a cellular origin using the presented method would assume that replication is bidirectional. This is true for the vast majority of chromosomal origins examined (see 2). It must be considered, however, that replication at some origins could be unidirectional or that pausing could occur (11). Once an origin is located approximately, the system presented here can be used to determine directionality, and, if replication is unidirectional, to map more precisely the start region. The task of making primers is obviously dependent on the amount of sequence information known.

The plots of Fig 6 reveal one complexity of the hybridization data of Fig 3. In the plots the ascending portions of each discontinuous curve do not proceed directly to infinity, as might be expected. This is due to the fact that on the slot blot of Fig 3 hybridization does not drop precipitously to zero for those nascent strands shorter than the length which should contain the probed amplified sequence. This is due primarily to the imperfect separation of DNA on this linear size gradient. Gradients with different configuration would alleviate this limitation, but more efficient size separation techniques should be sought.

One great advantage of the system developed here is that is does not require that any sequence comprising the origin or restriction sites surrounding it be known. One limitation of previously-developed methods is that restriction sites must be known in the vicinity of a potential origin (15,17,22). Conversely, a limitation of our method is that some sequence must be known at least within 10 kb of a potential origin. It should be noted that our method will apply quite well to mapping any individual origin the sequence for which may be highly repeated in the genome, provided that the active origin to be mapped is near a known, unique sequence. For example, it is conceivable that initiation could occur *in vivo* at loci containing interspersed, repetitive sequences including human Alu-family sequences (20). Initiation at particular such loci could be mapped with this procedure. It should also be noted that with our method the same size-fractionated preparation of nascent DNA can be used to screen a variety of different origin sequences.

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