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An Initiation Zone of Chromosomal DNA Replication Located Upstream of the c-*myc* Gene in Proliferating HeLa Cells†

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Studies on origins of DNA replication in mammalian cells have long been hampered by a lack of methods sensitive enough for the localization of such origins in chromosomal DNA. We have employed a new method for mapping origins, based on polymerase chain reaction amplification of nascent strand segments, to examine replication initiated in vivo near the c-*myc* gene in human cells. Nascent DNA, pulse-labeled in unsynchronized HeLa cells, was size fractionated and purified by immunoprecipitation with anti-bromodeoxyuridine antibodies. Lengths of the nascent strands that allow polymerase chain reaction amplification were determined by hybridization to probes homologous to amplified segments and used to calculate the position of the origin. We found that DNA replication through the c-*myc* gene initiates in a zone centered approximately 1.5 kilobases upstream of exon I. Replication proceeds bidirectionally from the origin, as indicated by comparison of hybridization patterns for three amplified segments. The initiation zone includes segments of the c-*myc* locus previously reported to drive autonomous replication of plasmids in human cells.

In mammalian chromosomes, DNA replicates as multiple replication units, termed replicons, activated at different times throughout S phase of the cell cycle (for a review, see reference 12). In each replicon, initiation occurs at a site called the origin of replication, and nascent DNA in each resulting replication bubble is elongated bidirectionally (12, 14). It has been shown that replication initiates in bacteria, lower eucaryotes, and animal viruses within preferred origin regions containing specific sequence elements (8; for a review, see reference 5). Very little is known, however, about origin specificity in mammalian cells. Identification of mammalian origins has been hampered so far by the lack of methods for mapping, isolation, or functional testing of chromosomal initiation sites. This is in large part due to the complexity of the mammalian genome, which necessitates extremely sensitive techniques for detection of replicating intermediates containing a single-copy DNA sequence. Existing procedures for mapping replication origins, based on two-dimensional electrophoretic analysis of replication intermediates, have localized initiation sequences in circular viral and plasmid molecules (11, 15, 23) and in yeast (*Saccharomyces cerevisiae*) cell chromosomal DNA (3, 16, 21), but these procedures are not practical for the more complex mammalian genomes. A recent mapping approach, based on the assumption that nucleosomes segregate conservatively at replication forks, has revealed the presence of an origin of replication near the human adenine phosphoribosyltransferase gene (13). This method also locates an origin in a region near the hamster dihydrofolate reductase gene overlapping previously reported initiation zones of the amplified dihydrofolate reductase locus (4, 20). However, this method does not allow precise localization, since sensitivity is reduced as one approaches within several thousand base pairs of an origin (13).

Recently, we have developed an origin-mapping method that overcomes most of the difficulties imposed by the complexity of mammalian genomes (25). This method utilizes antibodies to bromodeoxyuridine (BrdU) to isolate essentially pure, nascent, BrdU-labeled DNA strands and derives sensitivity from a polymerase chain reaction (PCR) amplification step. It has recently been demonstrated that this technique accurately maps a known DNA initiation site, that of simian virus 40 (SV40) DNA replicating in monkey CV-1 cells (25). Here, we describe the application of the PCR-based mapping method to location of an origin of chromosomal DNA replication near the human c-*myc* gene. Identification of this origin is of particular significance in relation to several recent reports concerning the replication of this gene.

The c-*myc* gene is one of the most thoroughly characterized proto-oncogenes in terms of its regulatory sequences and chromosome organization (10). Studies of polarity of DNA replication, based on in vitro runoff replication experiments, have indicated that replication forks progress through the gene in the direction of transcription (19). The c-*myc* gene is selectively amplified in certain tumor cells (1, 7, 27), and circular DNA episomes containing the c-*myc* gene can replicate autonomously (27). It has been reported that sequences 5′ of the c-*myc* gene can confer autonomous replication activity on plasmids transfected into human HeLa (22) or HL60 (17) cells. We report here the localization of an origin of chromosomal DNA replication within a 2-kilobase (kb) initiation zone centered approximately 1.5-kb upstream of the first exon of the c-*myc* proto-oncogene in HeLa cells.

MATERIALS AND METHODS

Cells, labeling, and isolation of DNA. Suspension cultures of HeLa cells were grown in Spinner flasks with Joklik-modified minimal essential medium (Sigma Chemical Co.) supplemented with 10% fetal calf serum. Cells (1×10^8 to 2×10^8 cells per 200 ml) were labeled with [^{3}H]deoxycytidine (3.0 μCi/ml; 19 Ci/mmol) and 5-BrdU (20 μM) for 10 min. All subsequent steps were performed in the dark or with an

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orange safety light to prevent light-induced damage to nascent BrdU-DNA strands. Cells were collected by centrifugation, washed with phosphate-buffered saline and lysed in 100 ml of 0.5% sodium dodecyl sulfate in 1.0 M NaCl–10 mM EDTA–50 mM Tris hydrochloride (pH 8.0). After overnight incubation with proteinase K (0.2 mg/ml), DNA was isolated by phenol-chloroform extraction and spooling from 70% ethanol.

Size fractionation and purification of nascent DNA strands labeled with BrdU. The labeled, high-molecular-weight DNA, isolated as described above, was dissolved in 0.2 M NaOH and layered onto three 5 to 20% linear sucrose gradients containing 0.2 M NaOH and 2.0 mM EDTA. Centrifugation was for 18 h at 15°C in a Beckman SW27 rotor at 24,000 rpm. Gradients were collected in 13 fractions and portions were taken to assay for ^{3}H radioactivity. Fractions 3 through 12 (designated here 1 through 10) were each neutralized with 2 N HCl in the presence of 0.1 M Tris hydrochloride (pH 8.0) and precipitated with ethanol. DNA was dissolved in 0.5 ml of 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA and subjected to two rounds of immunoprecipitation with anti-BrdU antibodies as previously described (25, 26), with the addition of salmon sperm DNA (200 μg per fraction; hydrolyzed by alkaline boiling) to each fraction to serve as carrier. Monoclonal anti-BrdU antibody (50 to 80 μl; Becton-Dickinson and Co.) was added to precipitate each fraction. After the first centrifugation, the pellets were washed with 0.5 ml of 0.15 M NaCl–10 mM sodium phosphate (pH 7.0) containing 0.05% Triton X-100. Pellets were deproteinized by overnight incubation in 200 μl of proteinase K in 0.5% sodium dodecyl sulfate with 10 mM EDTA and 50 mM Tris hydrochloride (pH 8.0), followed by phenol-chloroform extraction. After addition of 200 μg of new carrier DNA, the DNA of each fraction was ethanol precipitated and the immunoprecipitation cycle was repeated. Following the second round of immunoprecipitation and purification, samples were precipitated with ethanol in the presence of 20 μg of added *Escherichia coli* tRNA and redissolved in 50 μl of Tris-EDTA. DNA concentration in the purified, nascent DNA fractions was measured by fluorimetry with Hoechst 33258 dye in a Hoeffer DNA Minifluorimeter. Lengths of nascent DNA in each fraction were determined by electrophoresis on an alkaline 0.8% agarose gel in parallel with a denatured DNA marker ladder with 1-kb increments (Bethesda Research Laboratories, Inc.). Markers were visualized by ethidium bromide staining after neutralization. Nascent DNA sizes were determined by cutting each lane into 0.5-cm slices, melting the agarose by heating in 0.1 N HCl, and assaying for ^{3}H radioactivity by scintillation spectrometry.

Oligonucleotide synthesis and PCR amplification of nascent strand segments. Oligonucleotide primers and probes homologous to selected regions of the human c-*myc* locus were chemically synthesized by the phosphoramidite method as described previously (25). The nucleotide positions of each primer pair, numbered from the *Hin*dIII site, are as follows: A, 173 to 192 and 387 to 406; B, 947 to 966 and 1181 to 1200; C, 4463 to 4482 and 4668 to 4687; and D, 5951 to 5970 and 6179 to 6198. The positions of the probes are A, 200 to 220; B, 1031 to 1051; C, 4620 to 4640; and D, 5980 to 6000. One-fifth of the total DNA immunoprecipitated from each gradient fraction and redissolved as described above was used for PCR amplification with the indicated primer sets under conditions previously described (25). Amounts of DNA in each PCR reaction ranged from 5 ng in the shortest fractions to 50 ng in the longest fractions. Each PCR reaction of a gradient fraction involved coamplification of either two or three segments as described in the figure legends.

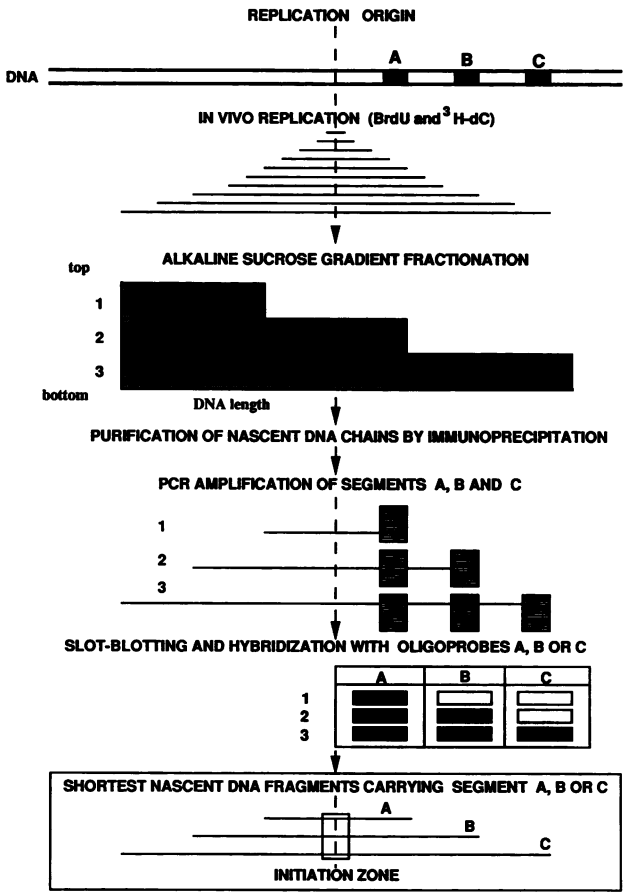

FIG. 1. General approach to mapping origins of replication by using PCR amplification of nascent strand segments. For any genomic DNA region of known sequence, three segments (A, B, and C) were chosen for PCR amplification. After pulse-labeling cultured cells with BrdU and [^{3}H]deoxycytidine as described in Materials and Methods, DNA strands were denatured and size fractionated by alkaline sucrose gradient centrifugation, and nascent strands were isolated by immunoprecipitation with anti-BrdU antibodies. After PCR amplification was performed on each separated strand fraction, slot blotting and hybridization revealed the fraction containing the shortest length of nascent DNA that includes each segment. On the basis of the size distribution of DNA within this fraction, a zone of initiation can be determined.

Slot blotting and hybridization. One-third of the DNA from each PCR reaction was slot blotted to a GeneScreen Plus membrane (Dupont, NEN Research Products) and hybridized to ^{32}P-end-labeled probe A, B, C, or D. Labeling, hybridization, autoradiography, and densitometric scanning of autoradiographs were all as described previously (25). Controls for PCR reactions employed total HeLa cell genomic DNA amplified with either individual pairs of primers or with coincubated primer sets and hybridized with the labeled probes as indicated in the figure legends.

RESULTS AND DISCUSSION

Mapping origins of DNA replication by using PCR amplification of nascent strand segments. The PCR-based method employed here for origin mapping is summarized in Fig. 1.

For any genomic DNA region of known sequence, three relatively short segments (A, B, and C) can be chosen for PCR amplification. This involves synthesis of three pairs of oligonucleotide probes as previously described (25). In unsynchronized cells, nascent DNA (i.e., newly synthesized DNA within a growing replication bubble) initiated at any given origin of replication will comprise a population of strands of all lengths extending outward from that origin. After pulse-labeling with BrdU and deoxycytidine, DNA strands can be denatured and size fractionated by alkaline sucrose gradient centrifugation, and nascent strands can be isolated by immunoprecipitation with anti-BrdU antibodies. If initiation takes place at a specific origin, then lengthening strands will progressively include the three chosen segments. PCR amplification can then be performed on each separated strand fraction with each of the three primer sets. Slot blotting and hybridization then reveals the fraction containing the shortest length of nascent DNA that includes each segment. Upon plotting the data graphically, as described below, a zone containing potential initiation sites is defined.

Localization of a replication origin in the human c-*myc* gene locus. Four PCR amplification segments, spanning about 6 kb of the c-*myc* locus, were selected from available sequence information (2) and chemically synthesized. To check the efficiency of the amplification reactions and the quality of the PCR products, a set of control reactions were performed with primers for each PCR segment and total HeLa DNA as a template. Thirty cycles of PCR produced well-defined agarose gel bands corresponding to the sizes of the selected PCR segments (not shown). Nonspecific hybridization was not detected under our conditions. Control reactions show that after coamplification, for each of three given c-*myc* segments, PCR amplification of 30 cycles yields product proportional to the original template concentration (Fig. 2). For segments A through C, the PCR reaction is linear in the template range used for this study (Fig. 2B). Thus, several-fold differences in the template concentration can readily be detected.

To label nascent DNA strands, unsynchronized proliferating HeLa cells were incubated for 10 min with [^{3}H]deoxycytidine in the presence of BrdU. High-molecular-weight DNA was then isolated, alkali denatured, and fractionated according to strand length by alkaline sucrose gradient centrifugation. ^{3}H radioactivity was determined for portions of each gradient fraction. The level of ^{3}H incorporation increased proportionally with DNA length, and the ^{3}H/DNA-length ratio was approximately constant for all gradient fractions, as would be expected for uniformly labeled, size-fractionated DNA. After neutralization and ethanol precipitation, nascent DNA in each size fraction was purified by two successive rounds of precipitation with an anti-BrdU monoclonal antibody. It has previously been shown that commercially available monoclonal antibodies against BrdU can efficiently purify a minor fraction of BrdU-DNA from a great excess of contaminating total DNA through immunoprecipitation (26). One round of immunoprecipitation has been successful in purification of nascent SV40 DNA present at a concentration of a single-copy DNA in human genomic DNA (25). In order to isolate nascent DNA copies of a single-copy human DNA, however, one must achieve a 20- to 50-fold higher level of purification to compensate for the difference in concentration of replicative intermediates between SV40 and human DNA. Therefore, a second independent round of immunoprecipitation in the presence of excess carrier DNA is required to reduce

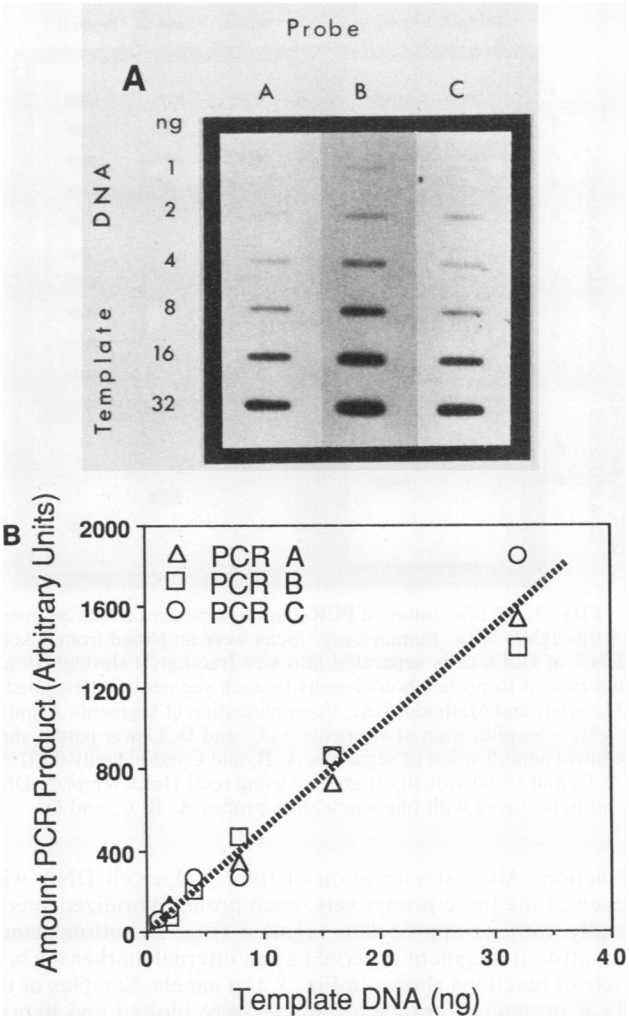

FIG. 2. PCR amplification of DNA segments of the c-*myc* locus as a function of genomic DNA template concentration. The indicated amounts of total HeLa cell DNA were added to a standard 100-μl reaction mix and amplified by using all six PCR primers for segments A, B, and C together under conditions described in Materials and Methods. Portions of the products were slot blotted and hybridized with probes specific for A, B, or C, respectively. Autoradiographs (A) were scanned, and the surface areas of the peaks were calculated. Values were normalized and plotted versus template DNA concentration (B). ⁞⁞⁞⁞, theoretically expected proportionality.

non-BrdU-substituted impurities to an insignificant level. This was demonstrated by control experiments in which BrdU-labeled HeLa DNA was premixed with highly labeled non-BrdU plasmid DNA and immunoprecipitated under our experimental conditions. The amount of labeled contaminating DNA recovered in the first pellet averaged 0.5% of the total, and this was reduced to <0.0004% by one additional round of purification.

Samples of each immunopurified nascent DNA fraction were amplified by PCR in the presence of primer sets either for coamplification of segments A and B or for coamplification of segments A, C, and D. This internally controlled amplification eliminates effects of possible fluctuations in reaction conditions and allows more accurate calculation of relative differences in template concentrations for each

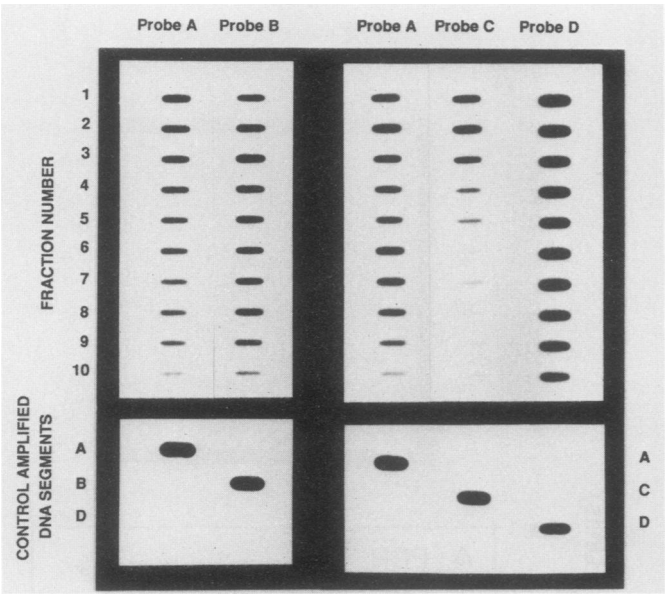

FIG. 3. Hybridization of PCR-amplified nascent DNA. Segments A through D of the human c-*myc* locus were amplified from nascent DNA of HeLa cells separated into size fractions 1 through 10 and hybridized to probes homologous to each segment as described in Materials and Methods. Left, Coamplification of segments A and B; right, coamplification of segments A, C, and D; Lower panels show control amplification of segments A, B, and C individually (left) and A, C, and D individually (right) by using total HeLa template DNA and hybridizing with oligonucleotide probes A, B, C, and D.

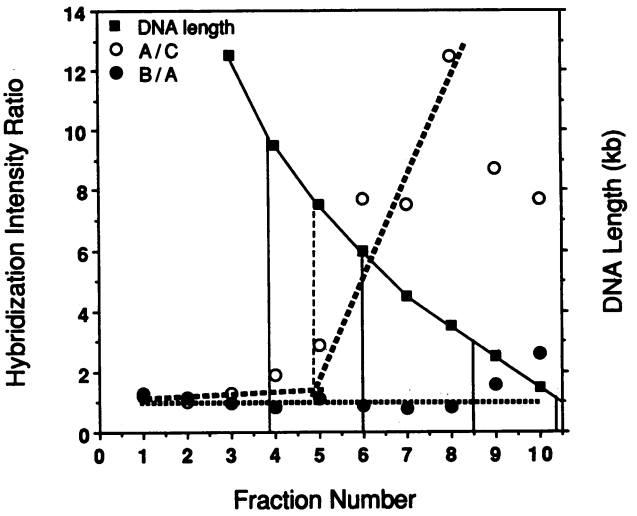

FIG. 4. Hybridization intensity ratios of PCR-amplified HeLa DNA fractions. Autoradiographs of the slot blot hybridization (Fig. 2A) were densitometrically scanned as described in Materials and Methods and the areas of the peaks were determined. After normalization of the signal for the longest DNA fractions, 1 through 3, the ratios of areas B/A (●) and A/C (○) for each DNA size fraction were plotted versus fraction number. Lines for these ratios were plotted by linear regression analysis as described in the text. The average sizes of nascent DNA fragments in each fraction (■) are also plotted. The vertical dashed line shows the projection of the inflection point of the A/C ratio and the corresponding DNA size. The inflection point for B/A cannot be calculated precisely, but its estimated error interval is indicated. Solid lines show the error intervals (±1 gradient fraction) and their corresponding DNA sizes. Lengths of nascent DNA in each fraction were determined by electrophoresis on an alkaline 0.8% agarose gel in parallel with a denatured DNA marker ladder with 1-kb increments (Bethesda Research Laboratories).

fraction. After amplification of total HeLa cell DNA with each of the three primer sets, each probe hybridized specifically with its appropriate segment (Fig. 3, bottom panels [controls]). Segment A served as an internal marker for both sets of reactions shown in Fig. 3, top panels. Samples of the PCR products from each fraction were blotted and hybridized with oligonucleotide probe A, B, C, or D. The hybridization patterns obtained (Fig. 3) show that segments A and B are present in nascent DNA from most of the size fractions but that A decreases relative to B in fractions 9 and 10. Segment C could be amplified equally well from the DNA of fractions 1 through 3 but decreased after fraction 3. The patterns obtained are consistent with location of a chromosomal replication initiation site near both segments A and B. In that case, segment C would be present only in the fractions with sizes greater than twice the distance from the origin to the distal end of the segment (Fig. 1). Localizing the origin downstream of segment C would not be consistent with the hybridization patterns of segments A and B and, thus, such a location of origin is unlikely. An unusual hybridization pattern was observed for segment D. It most likely represents an artifact which will be discussed in detail below. To determine the distances from the PCR segments to the potential origin, ratios of densitometric scans of the slot blots for each segment were calculated and plotted as in Fig. 4. B/A segment ratios are nearly constant and near 1 for fractions 1 through 8 and increase to approximately threefold in fraction 10. This would be the result if both segments are near a replication origin which is closer to B than to A. The A/C ratios for fractions 1 through 8 form a pattern readily plotted as two linear components by regression analysis. The linear component comprising fractions 1 through 4 is parallel to and has approximately the same intercepts as the line for B/A. The second regression component, for fractions 5 through 8, reflects the dramatic decrease in concentration of segment C in size classes shorter than fraction 4. Note that the shortest fractions, 9 and 10, are not included in the linear regression for the A/C ratio in Fig 4. The primary reason for not including these fractions is that the ratio for these points is affected by the decrease seen in relative concentration of amplified segment A (Fig. 3; Fig. 4, B/A ratio). The A/C ratio is presented here instead of B/C because segments A and C were coamplified in the same reaction, as shown in Fig. 3. Because of the 1:1 ratio of B to A for most of the fractions, the B/C ratio yields the same inflection point near fraction 5 as does A/C. The two linear components of A/C intersect at a point indicating the shortest nascent DNA strands that include segment C. This point corresponds to a size of 7.8 kb. If replication at this origin is bidirectional and symmetrical, the origin would be located in the center of this segment, i.e., at a point 3.9 kb upstream of the 3′ end of segment C. This value represents the midpoint of an error interval designated as an initiation zone in Fig. 1. How large is this error interval? According to our experimental results (Fig. 4), the inflection point occurs near fraction 5. The corresponding maximum error interval (±1 gradient fraction) would be 6 to 10 kb (Fig. 4), and the origin of bidirectional replication would be located between 3 and 5 kb from the 3′ end of segment C. Similar analysis of the B/A ratio (Fig. 4) shows a transition near fraction 10. Although this transition, close to the end of the gradient, does not allow for a linear regression analysis, another approximate

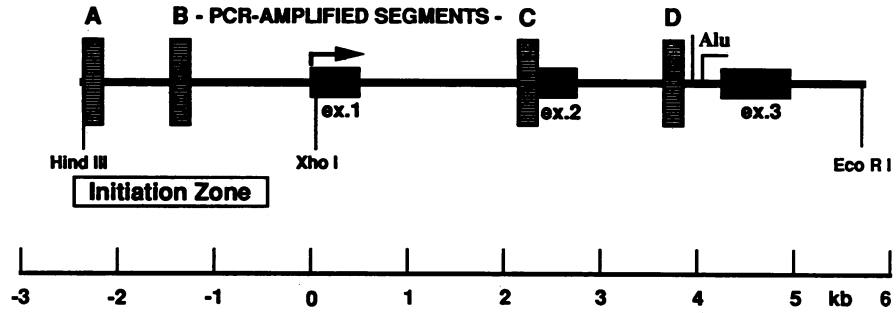

FIG. 5. Mapping an origin of chromosomal DNA replication near the HeLa c-*myc* locus. The 8.1-kb *Hin*dIII-*Eco*RI genomic DNA fragment is shown. ex., Exon. The arrow shows the c-*myc* gene P_1 promoter and direction of transcription. Shaded boxes A, B, C, and D indicate the positions of PCR-amplified segments used for mapping. The Initiation Zone below indicates the error interval within which an origin of bidirectional DNA replication is located on the basis of the DNA length measurements shown in Fig. 4.

error interval can be estimated which places the initiation site 0.5 to 1.5 kb downstream of the 5′ end of segment A, within the interval projected by the A/C ratio. These intervals are represented as an initiation zone in Fig. 5. An additional probe position (segment E; 2,948 to 3,190 nucleotides from the *Hin*dIII site; located just 3′ to exon I) was also examined. The B/E ratio coincides largely with that of B/A and is not plotted separately, but it is also consistent with location of the initiation zone, as shown in Fig. 5. Although initiation could take place anywhere within the 2-kb initiation zone (Fig. 4 and 5), its probability is much higher near the center of the zone. This is in agreement with the hybridization patterns for segments A and B. More precise estimation of the initiation zone is limited by the size heterogeneity of sucrose gradient fractions. In theory, if an origin of replication is present near segment B, the A/C ratio would approach infinity for any nascent strand length that does not include segment C. Instead, a linear regression could be performed for A/C points (Fig. 4). This could be due in part to three factors. Firstly, each sucrose gradient fraction represents a distribution of sizes, with an average peak value as shown in Fig. 4. The fractions overlap in size, and this contributes to the appearance of a slope in the regression curve instead of a sharp transition. Secondly, partial nascent DNA breakdown products could be present in gradient fractions. Complexities arising from Okazaki fragments are minimized in the mapping procedure, as described previously (25). Thirdly, the observed A/C transition may reflect an intrinsic feature of the initiation process itself. If replication does not always initiate at exactly the same site, but rather within a broader region with a higher concentration of initiation events in its center, this would be reflected in a curve similar to that for A/C ratios in Fig. 4.

The 2-kb zone of replication initiation projected from Fig. 3 is centered approximately 1.5-kb upstream of the P_1 transcription initiation site (Fig. 5). This conclusion is based on a bidirectional mode of replication that is responsible for the vast majority of mammalian origins (5, 12, 14). However, a recent study has suggested preferential unidirectional replication as the mode for yeast ribosomal genes (21), possibly because of the positioning of replication fork barriers (3). The PCR method allows distinguishing between bidirectional and unidirectional replication for the c-*myc* replicon. Unidirectional replication of the c-*myc* locus would yield the observed hybridization pattern for probe C only if an origin is located more than 8 kb upstream of segment C. This, however, would yield a pattern different from those actually observed for probes A and B. These data, therefore, indicate that the c-*myc* origin initiates bidirectional replication and that forks progress in this fashion, at least so far as to include segment C.

Segment D yields an unusual hybridization pattern in that the overall level of amplified DNA is three- to sevenfold higher than that of segments A or B for all of the size fractions. This pattern is most likely due to annealing of repetitive genomic DNA sequences during the immunoprecipitation step in the isolation of nascent chains. It was previously observed that when nascent repetitive DNA sequences are to be purified from a great excess of unreplicated DNA carrying the same sequence, they may reassociate and form partial duplexes during incubation with the antibody (25). Although this is not a problem for single-copy mammalian sequences with very low C_0t values, it may occur when an amplified segment is at or near a repetitive sequence. Although it does not include *Alu* sequences, segment D is located within about 100 base pairs of an *Alu* family repeat. *Alu* sequences have been implicated as potential replication initiation sites in the presence of SV40 T antigen (18). It is unlikely, however, that the *Alu* repeat of c-*myc* intron II initiates standard bidirectional replication, since hybridization patterns of segments A through C do not reflect this. It is most likely that reannealing of *Alu* repeats, widely represented in the total nascent DNA, during immunoprecipitation has allowed inclusion of nonreplicated c-*myc* sequences, carrying segment D from the vicinity of the *Alu* repeat, with the precipitated nascent chains. One line of evidence for this is that after the immunoprecipitation step, the D/B ratio increases severalfold compared with A/B or C/B.

The chromosomal DNA replication initiation zone identified here coincides approximately with regions of autonomously replicating sequence (ARS) activity reported previously for plasmids transfected into HeLa cells. McWhinney and Leffak have observed ARS activity in a fragment extending 2.3 kb upstream of the P_1 promoter (22). Iguchi-Ariga et al. have reported an ARS activity in a fragment approximately 2.1 kb upstream of the promoter (17). Both of these sites are within the zone of chromosomal replication initiation projected by the data in Fig. 3. The zone also contains other elements implicated in replication in various systems. The zone is centered near multiple repeats of a pentanucleotide element essential for initiation at *Alu* repeats in transfected COS7 cells (18). The center of the zone is also near a yeast ARS consensus (5) and two DNase I hypersensitive sites (9). The initiation zone includes a consensus sequence for binding of nuclear factor 1 (a cellular protein required for adenovirus replication [24]). It has recently been reported that nuclear factor 1 stimulates

replication of SV40 minichromosomes in vivo and in vitro by a mechanism involving perturbation of chromatin structure (6). The relevance of these elements to replication of the c-*myc* gene is presently unknown.

The PCR mapping method described here allows for the first time the mapping of a mammalian replication origin to within a zone of approximately 2 kb in chromosomal DNA in vivo. The greatest advantage of the method is its high sensitivity, derived from PCR. An additional advantage is that the method does not require cell synchrony and is applicable to origins activated not only at the onset of but throughout the entire S phase. The method is based on the sizing of DNA strands synthesized under normal growing conditions in cells not perturbed by metabolic inhibitors or other agents. By selecting PCR amplification segments within unique sequences, one can overcome the difficulties imposed by repeated elements upon any mapping approach based on specific hybridization. For the most part, these advantages are not unique to eucaryotes, and this method of mapping origins may be applied to a wide range of replicating systems.

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