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Fine mapping of a replication origin of human DNA

(competitive polymerase chain reaction/DNA replication/lamin B2)

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ABSTRACT **A highly sensitive procedure was developed for the identification of the origin of bidirectional DNA synthesis in single-copy replicons of mammalian cells. The method, which does not require cell synchronization or permeabilization, entails the absolute quantification, by a competitive PCR procedure in newly synthesized DNA samples, of the abundance of neighboring DNA fragments distributed along a given genomic region. This procedure was utilized for mapping the start site of DNA replication in a 13.7-kb region of human chromosome 19 coding for lamin B2, which is replicated immediately after the onset of S phase in HL-60 cells. Within this region, DNA replication initiates in a 474-bp area corresponding to the 3′ noncoding end of the lamin B2 gene and the nontranscribed spacer between this gene and the 5′ end of another highly transcribed one. This localization was obtained both in aphidicolin-synchronized and in exponentially growing HL-60 cells.**

Living organisms regulate the rate of DNA replication by modulating the activation of replication origins (1, 2); in eukaryotes, every chromosome is composed of many tandemly organized replicons, which are activated at different times of the S phase (3) and are characterized by an origin from which two oppositely moving semiconservative forks issue. However, identification of replication origins in animal systems has proven very difficult in view of the widely experienced failure to isolate autonomously replicating sequences in mammalian cells (4–7). This failure has fostered several attempts to utilize physicochemical or biochemical approaches for the identification of origins. Analysis of leading strands polarity switch indicated the existence of an origin within 14 kb in the amplified dihydrofolate reductase (DHFR) locus of Chinese hamster cells (8, 9) and, more recently, within a 2-kb stretch of the human β-globin gene (10). PCR analysis of the relative abundance of different markers in isolated nascent DNA strands has given indications that the DHFR origin may be localized in a 2-kb region (11). Furthermore, analysis of the switch-point of polarity of the Okazaki fragments has pointed to an origin restricted within 450 bp of the same area (6). In contrast, the two-dimensional gel approach has given indications that replication initiates in delocalized fashion within a 50-kb origin area (12) in the same amplicon as well as within a 3-kb stretch near the *ura4* gene of *Schizosaccharomyces pombe* (13).

In view of the controversial nature of these results [for a critical discussion, see Falaschi *et al.* (14)], we have tried to identify a start site of DNA replication in human cells by developing a technique that (*i*) does not rely on amplified sequences, (*ii*) does not depend on possibly subjective interpretations of complex electrophoretic patterns, (*iii*) can be applied to intact cells without the need for permeabilization for labeled precursors, (*iv*) does not depend on the extensive use of metabolic inhibitors with possible pleiotropic effects, and (*v*) does not require *a priori* the synchronization of the cell culture.

MATERIALS AND METHODS

Cell Cultures. HL-60 and COS-1 cells were cultured in RPMI 1640 medium and Dulbecco's modified Eagle medium, respectively, as described (14, 15). HL-60 cells (2.5×10^8) were uniformly labeled for 3 days with [^{14}C]thymidine and synchronized at the G_1/S border with aphidicolin as described (16). After release from the block, newly synthesized DNA was labeled for 10, 20, or 30 min, in the presence of a subinhibitory concentration of aphidicolin (1 μg/ml), by the addition of 1 μM (final concentration) [^{3}H]deoxycytidine (21.5 Ci/mmol, Amersham; 1 Ci = 37 GBq) and 100 μM (final concentration) unlabeled BrdUrd (Boehringer Mannheim). In the experiments with asynchronous cells, 2.2×10^8 cells were pulse labeled for 10 min as above; in the control experiment shown in Fig. 4*B*/*b* labeling was continued for 24 hr.

Terminal differentiation of HL-60 was achieved with retinoic acid and dimethylformamide, as described (17).

Transfection. Plasmid pAWTSV (≈9 kb), a kind gift of Cesare Vesco (Institute of Cell Biology, Rome), carries the whole simian virus 40 (SV40) genome inserted in the *Bam*HI site of pAT153 (18). Six 10-cm tissue culture plates, containing about 10^6 COS-1 cells each, were transfected with 10 μg of pAWTSV by the calcium phosphate precipitation technique. After 10 hr of incubation in calcium phosphate solution, cells were extensively washed and fresh medium was added, containing 10 nCi of [^{14}C]thymidine per ml. After 18 hr of incubation, BrdUrd (100 μM final concentration) and [^{3}H]deoxycytidine (1 μM final concentration) were added. After 1 min of incubation, cells were killed by addition of sodium azide and DNA was extracted as described below.

Extraction and Purification of Newly Synthesized DNA. Total DNA was extracted, denatured, and size-fractionated by sedimentation through neutral sucrose gradients as described (15).

In the experiment involving transfection of plasmid pAWTSV, DNA (700 μl final volume) was fractionated on four 5–20% (wt/vol) linear sucrose gradients (5 ml each) for 210 min at 20°C in a Beckman SW55Ti rotor at 55 krpm; 24 fractions of 200 μl were collected.

In the experiment with synchronized HL-60 cells, DNA (2 ml final volume) was fractionated on eight 5–30% sucrose

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Abbreviations: DHFR, dihydrofolate reductase; SV40, simian virus 40.

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gradients (5 ml each) for 5 hr at 20°C in a Beckman SW55Ti rotor at 45 krpm; 24 fractions of 200 μl were collected.

In the experiments with asynchronous cells, DNA (8.8 ml final volume) was size fractionated on four 5–30% sucrose gradients (38 ml each) for 20 hr at 20°C in a Beckman SW28 rotor at 26 krpm; 12 fractions of 3 ml were collected.

The corresponding fractions from the parallel gradients of each of the above-described experiments were combined; fractions were pooled as described for each experiment, dialyzed, and further purified by anti-BrdUrd immunoaffinity chromatography (19).

In the control experiment of Fig. 4*B*/*b*, DNA was extracted, sonicated at an average size of 1000 bp, heat-denatured, and submitted to the same anti-BrdUrd affinity chromatography purification procedure as the samples above.

PCR Amplification and Competitor Construction. For each DNA region to be amplified, four primers were synthesized. The first two (external) primers of each set were chosen in order to amplify DNA fragments of 100–300 bp with an AT content ranging from 32% to 63%; the last two primers of each set consist of two common 5′ tails of 20 nt (unrelated to genomic sequences; tail1: 5′-ACCTGCAGGGATCCGTCGAC-3′; tail2: 5′-GTCGACGGATCCCTGCAGGT-3′) linked to specific sequences complementary to genomic targets on the 3′ end; they were utilized for the construction of competitors according to a previously described procedure (20). These competitors have the same sequence as the genomic targets, except for an addition of 20 nt in the middle; they were quantified by coamplification with a known amount of plasmid DNA (for the SV40 experiments) or total genomic DNA (for the human DNA experiments).

The names and localizations (in parentheses) of the oligonucleotides utilized (relative to the GenBank file humlamb2b for sets BE2, B48 TER, BN1, and B48BIS; to file humins01 for INSULIN; to file sv4cg for, SV5, SV14, SV13, and SV12) were as follows. Region BE2: primers BESX (2500–2481), BE2DX(II) (2327–2308), BE2+/1 (tail1+2409–2390), BE2+/2 (tail2+2410–2429). Region BN1: primers BN1SX (3421–3402), BN1DX (3217–3236), BN1+/1 (tail1+3328–3309), BN1+/2 (tail2+3329–3348). Region B48BIS: primers B48BISSX (3822–3803), B48BISDX (3643–3662), B48BIS+/1 (tail1+3726–3707), B48BIS+/2 (tail2+3727–3746). Region B48TER: primers B48TERSX (4502–4483), B48TERDX (4298–4317), B48TER+/1 (tail1+4402–4383), B48TER+/2 (tail2+4403–4422). Region INSULIN: primers I-SX (2491–2510), I-DX (2631–2612), I+/1 (tail1+2559–2578), I+/2 (tail2+2558–2539). Region SV5: primers SV5SX (1921–1940), SV5DX (2251–2232), SV5/C−(tail1+2072–2091), SV5/C+ (tail2+3727–3746). Region SV13: primers SV13SX (597–616), SV13DX (855–836), 13 per DX (tail+750–769), 13 per SX (tail2+749–730). Region SV12: primers SV12SX (4740–4759), SV12DX (5041–5022), 12 per DX (tail1+4940–4959), 12 per SX (tail2+4939–4920). Region SV14: primers SV14SX (2944–2963), SV14DX (3225–3206), 14 per DX (tail1+3033–3052), 14 per SX (tail2+3032–3013). Primers and competitors for the SB12, SE10, B13, B48, SE17, and β-globin regions have already been described (15, 20). The PCR cycle profiles were as follows: denaturation at 94°C; annealing at 56°C for the primer sets INSULIN and SV13, 60°C for BE2, B48BIS, and BN1, 42°C for SV5, SV12, and SV14, and 64°C for B48TER; extension at 72°C; time for each step was 30 sec; 50 cycles were performed with 2.5 units of *Taq* polymerase (Perkin–Elmer/Cetus), according to the conditions recommended by the manufacturers.

Competitive PCR experiments with newly synthesized DNA samples were carried out by a two-step procedure, by challenging the same amount of BrdUrd-substituted DNA

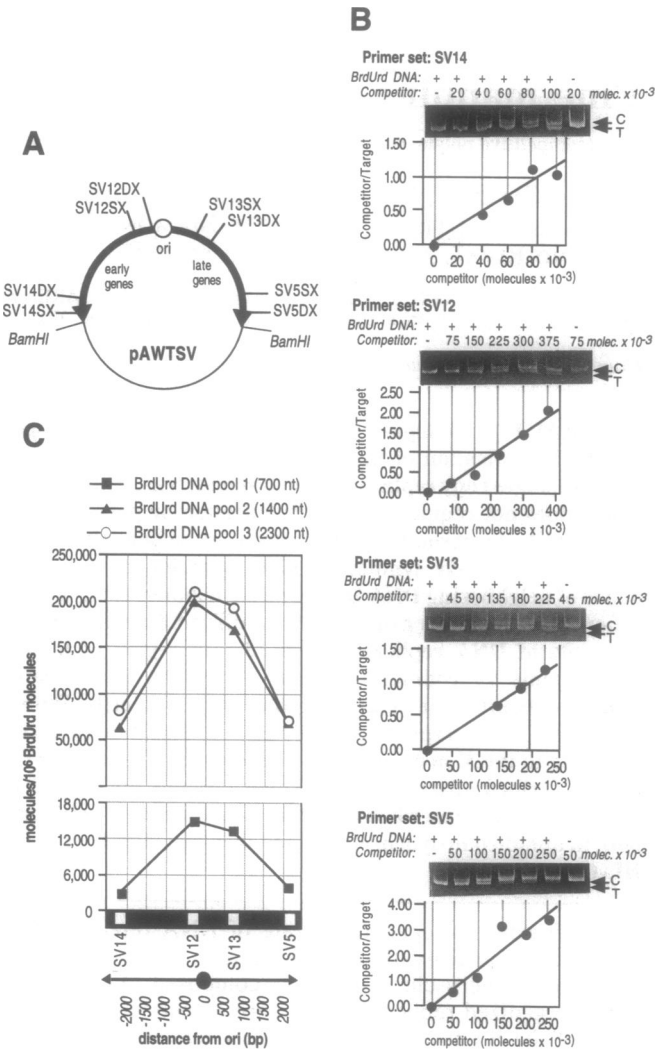

FIG. 1. Competitive PCR for origin mapping: SV40 model. DNA replication of plasmid pAWTSV was studied after transfection of T-antigen-expressing COS-1 cells. Eighteen hours after transfection, newly synthesized DNA was extracted, denatured, and size-fractionated on sucrose gradients. Three pools of 700, 1400, and 2300 nt were collected and further purified. The relative abundance of the four indicated regions of SV40 DNA was tested in the three nascent DNA pools by competitive PCR. (*A*) Schematic representation of plasmid pAWTSV. The SV40 portion of the plasmid is indicated by the thick line; the arrows indicate the direction of late and early gene transcription. The position of the origin of replication (ori) is indicated by a circle. The positions of the primer pairs used are shown. (*B*) Competitive PCR experiments for the quantitation of SV40 molecules in BrdUrd-substituted DNA pool 3 (2300 nt). For each primer set, a fixed amount of the newly synthesized DNA sample was mixed with increasing amounts of the corresponding competitors and amplified. The products obtained (arrows: C, competitor; T, target) were resolved by polyacrylamide gel electrophoresis, stained with ethidium bromide, and quantified by scanner densitometry. The ratio between the upper (competitor) and lower band (newly synthesized DNA) was then plotted against the number of molecules of competitor added to the reaction, and the best fit line was drawn. The amount of competitor interpolating at a 1:1 ratio between the two amplification products corresponds to the amount of BrdUrd-substituted molecules in the sample containing target sites for amplification. (*C*) Results of quantitation. For each sample of newly synthesized DNA, the relative abundance of molecules corresponding to the four investigated regions of SV40 is plotted against the relative distance of these regions from the origin of replication (position 0 on the map). The regions on the early gene side are arbitrarily indicated by negative numbers. The number of BrdUrd-substituted DNA molecules detected by each primer pair is expressed as molecules per 10^6 total BrdUrd-substituted molecules of the same size, estimated on the basis of specific radioactivity.

first with 10-fold dilutions and subsequently with 2-fold dilutions of each competitor (20).

RESULTS AND DISCUSSION

Procedure for Origin Mapping. An origin of bidirectional DNA replication in cultured cells is pulse-labeled with BrdUrd for different times and the labeled DNA is then isolated, denatured, and size fractionated. The newly replicated DNA molecules near an origin are much smaller than the bulk of DNA and thus markedly enriched in the low molecular weight DNA fraction. This BrdUrd-labeled fractionated DNA is further affinity purified through a column containing an immobilized monoclonal antibody against BrdUrd-substituted DNA. The bound fragments are then eluted and assayed quantitatively for the presence of sequences localized in adjacent positions on the chromosome. The relative abundance of the different sequences should give a distribution of values peaking at the replication start site. A similar approach was adopted to tentatively localize the origins of the DHFR and c-*myc* loci (11, 21), but its outcome suffers from the poor quantitative reproducibility of the conventional PCR technique.

To attain the necessary sensitivity and precision, we have established a quantitative PCR method (20) based on competition for the selected primers between the genomic target and a competitor molecule differing from the genomic sequence only for an insertion of 20 bp in the middle.

Competitive PCR for SV40 Origin Mapping. The technique was first validated on the SV40 origin of DNA replication. For this purpose, DNA competitors were prepared for four SV40 DNA regions mapping at different distances from the origin. Plasmid pAWTSV, containing the whole SV40 genome cloned in a prokaryotic vector (Fig. 1*A*), was transfected into COS-1 cells chronically labeled with [^{14}C]thymidine and, 18 hr later, the cells were pulse-labeled for 10 min with BrdUrd and the DNA was extracted and processed as outlined above. Following sucrose gradient fractionation, three pools of single-stranded, BrdUrd-labeled DNA of 700, 1400, and 2300 nt were selected. The choice of sizes is crucial, since smaller sizes would be enriched in Okazaki fragments (and hence scattered all along the chromosome), whereas bigger sizes would be too close to the size of the whole genome (for the plasmid) or to the area of the gradient containing the bulk of the DNA (for the chromosomal origins, see below). For these reasons, this and all subsequent experiments were performed with newly synthesized DNA fragments ranging in size between 600 and 2500 nt.

The competitive PCR experiment for the 2300-nt sample is depicted in Fig. 1*B*. Results for all three fragment sizes, expressed as the number of specific molecules per 10^6 total molecules of the same size (Fig. 1*C*), confirm that, indeed, the sequences close to the origin are markedly enriched over those distant from it, at all selected sizes. Since the size of the SV40-based replicon is much smaller than that of a human replicon, this test is particularly stringent.

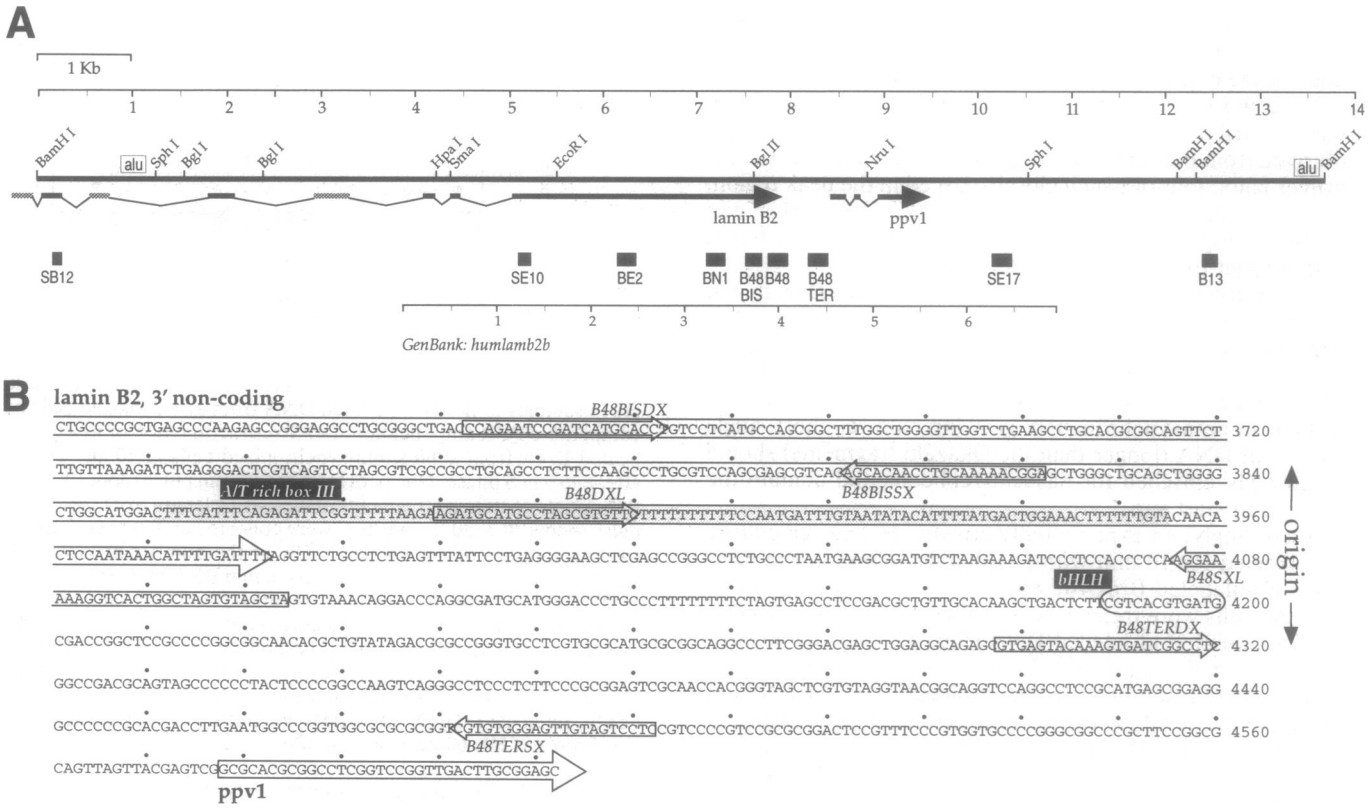

FIG. 2. Schematic representation of the human genomic region analyzed in this study and sequence of the origin area. (*A*) The 13.7-kb region of human chromosome 19 investigated contains the 3′ end of the gene encoding lamin B2 and another tandemly arranged small gene (ppv1). The intron–exon distribution of the two transcripts is shown; the exons represented with a gray line were not precisely localized. The positions of the segments amplified by PCR are indicated by the black boxes at the bottom. The sequence of the core portion of this region (humlamb2b) is available in GenBank (accession no. M94363). (*B*) Sequence of the region encompassing the three neighboring markers giving the highest enrichment in the quantitation experiments of Figs. 3 and 4 (B48, B48BIS, and B48TER). Locations of the primers used for the amplification are indicated by empty boxes with arrowheads at the 3′ end. Primers B48BISSX and B48TERDX flank the start site of DNA replication. The wider empty arrows indicate the transcripts. The (A+T)-rich tract in the lamin B2 transcript is indicated by gray shading (A/T-rich box III). The binding site for basic/helix–loop–helix family of proteins is shown.

Human DNA Origin in Synchronized Cell Cultures. In the previous years, we demonstrated that a 1560-bp DNA fragment [B48, selected from a mini-library of HL-60 cell DNA containing fragments derived from the replicons activated at the beginning of the S phase (16)] was in fact replicated in the very first minute of the S phase in HL-60 synchronized cells (22) and, thus, presumably very close to, or even coincident with, an origin. We sequenced 13.7 kb around the initial isolate, and we observed that this genomic region, mapping on band p13.3 of chromosome 19, contains the final portion of the lamin B2 gene and, downstream of it, another gene (ppv1), transcribed in the same direction and coding for a still unidentified product [Fig. 2A (22)].

To map the origin within this region, we challenged HL-60 cells with the described approach by synchronizing a culture at the G1–S border and then allowing incorporation of BrdUrd for two different time intervals in the presence of a subinhibitory concentration of aphidicolin. After 10- and 20-min pulses (corresponding to approximately 8 and 16 sec of synthesis in the absence of aphidicolin), DNA was extracted, denatured, and fractionated on a sucrose gradient, and pools of approximately 1500 nt (from the 10-min pulse) and 600, 1400, and 2400 nt (from the 20-min pulse) were selected; these were subsequently purified on the anti-BrdUrd-DNA antibody column and analyzed for the abundance of the sequences indicated in Fig. 2*A*. The results are reported in Fig. 3 *A* and *B* and fit with the presence of a replication start site near the original isolate (B48 marker) for both pulse times and for all different size samples. These data confirm a preliminary mapping performed with a 10-min pulse with the same technique (15). As a control, we quantified in the same sample an unrelated DNA sequence [located within the β-globin gene (10)] that, as shown in Fig. 3*B*, gave values comparable to the lowest ones observed at the ends of the explored area.

For a more precise definition of the peak of marker abundance distribution, we challenged a 1000-nt BrdUrd DNA pool (derived from the same culture after a 30-min pulse) with nine primer pairs, four of which closely spaced in the peak region. Again, a sharp distribution peaking at the B48 marker was identified (Fig. 3*C*), this sequence being enriched over 10^4-fold with respect to a random sample of DNA.

Lamin B2 Origin in Asynchronous Cultures. Due to the accuracy and sensitivity of competitive PCR, the described approach is not restricted to synchronized cells but can be extended to exponentially growing ones, thus avoiding possible artefacts introduced by the synchronization procedures. In fact, in an asynchronously growing culture, short single strands of DNA (longer than the Okazaki fragments) should be strongly enriched for nascent replicons and contain the replication start site.

Accordingly, an exponentially growing HL-60 cell culture was labeled for 10 min with [^{3}H]deoxycytidine in the presence of unlabeled BrdUrd, without any other addition, and then the cells were treated as described above. As expected, the fractionation of labeled DNA (Fig. 4*A*) showed a distribution of small fragments of progressively increasing size, separated from the bulk of high molecular weight DNA. A DNA sample of ≈1500 nt was analyzed for the abundance of the nine markers indicated in Fig. 4*B*/*a*. Again, an unambiguous peak at the same position as observed in the previous experiment was obtained. The abundance of another origin-unrelated gene (insulin) in this sample was as low as or lower than that of the markers furthest from the origin peak.

From the data presented in Fig. 4 we can estimate that one molecule of B48 sequence is present every 30,000 size-fractionated and immunopurified BrdUrd-DNA fragments. This value is in line with the expected one, since in an asynchronous cell population all genome origins (roughly 30,000 assuming an average replicon size of 10^5 bp) should be activated at any given moment.

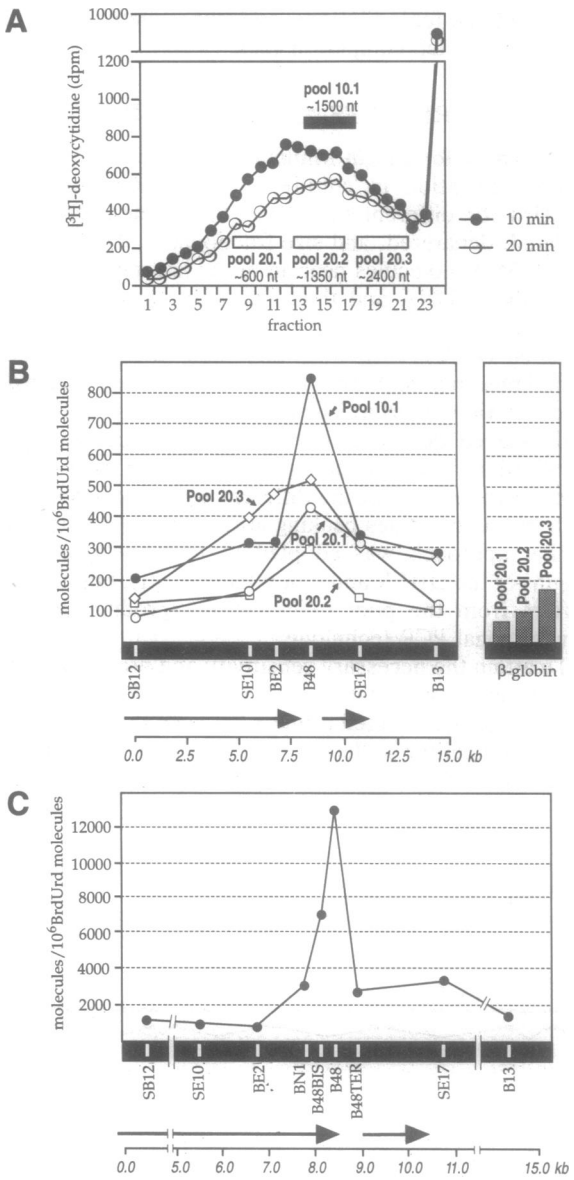

FIG. 3. Quantitation of the abundance of selected genomic markers in samples of newly replicated DNA from synchronized cells by competitive PCR amplification. (*A*) Size-fractionation of newly replicated DNA from aphidicolin-synchronized cells at 10 and 20 min after entry in S phase. One pool from the 10-min pulse (pool 10.1) and three pools containing fragments of increasing length from the 20-min pulse (pools 20.1, 20.2, and 20.3) were collected and further purified. (*B*) Quantitation by competitive PCR of the number of molecules containing markers scattered along the lamin B2 domain (see scheme on abscissa and Fig. 2) in the newly synthesized DNA samples of *A*. As a control, the number of molecules containing a segment of the β-globin gene in the same samples is shown on the right. (*C*) Precise mapping of the start site of DNA replication by quantitation of the abundance of nine markers along the lamin B2 region in a sample of newly replicated DNA of ≈1000 nt purified from aphidicolin-synchronized HL-60 cells 30 min after entry in S phase.

To rule out possible biases in the estimate of abundance of selected DNA fragments introduced by the density labeling and/or purification procedures, an HL-60 culture was uniformly labeled for 24 hr with BrdUrd; nuclear DNA was extracted, sonicated to ≈1000 bp, denatured, purified as above, and assayed for the abundance of five lamin B2 markers (Fig. 4*B*/*b*): no significant enrichment for any sequence (including B48) was observed.

As a further control that the primers used did not introduce any bias in the analysis of marker abundance, the relative

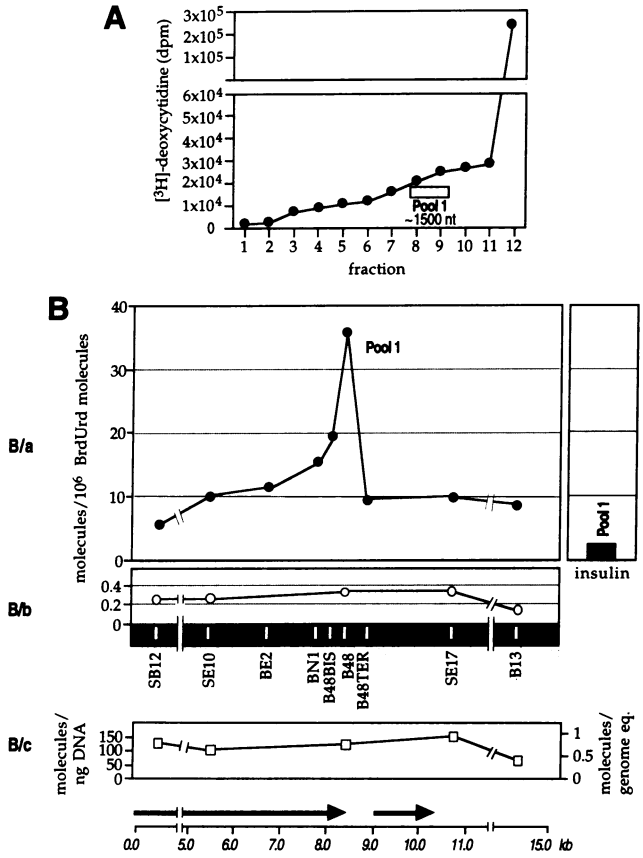

FIG. 4. Fine mapping of the start site of DNA replication in the lamin B2 gene domain in exponentially growing HL-60 cells. (*A*) Size-fractionation by sucrose gradient centrifugation of newly synthesized DNA from pulse-labeled HL-60 cells. (*B*) (*B/a*) Quantitation of the number of molecules containing different regions scattered in the lamin B2 gene domain within the DNA pool 1 (*A*). As a control, the number of molecules containing a segment of the human insulin gene is shown on the right. (*B/b*) Quantitation of the number of molecules within a sample of uniformly BrdUrd-substituted total genomic DNA, with an average fragmentation size of 1000 nt, submitted to the same procedure as the samples from *A*. (*B/c*) Quantitation of the number of molecules in a sample of total unsubstituted genomic DNA from differentiated HL-60 cells. In *B/c*, the number of molecules is expressed as molecules per ng of total DNA (left); conversion of this value to molecules per genomic equivalents (right) was achieved assuming that 1 genomic equivalent (haploid genome) corresponds to about 5.5 pg of DNA.

content for the same segments probed in this work was measured in a sample of unreplicating DNA from HL-60 cells differentiated by treatment with retinoic acid and dimethylformamide. As expected, in this sample (Fig. 4*B/c*) all different markers show an abundance close to one copy per genome.

Features of the Origin Region. The pair of primers (B48BIS and B48TER) flanking the one giving the strongest signal (B48, Fig. 2*B*) confine the replication origin within a 474-bp region roughly corresponding to the nontranscribed spacer between two transcription units arranged head to tail (22). This region was shown to contain a strong promoter for the downstream transcription unit; a key feature of this promoter is the presence of a basic helix–loop–helix protein binding motif that was shown to bind *in vitro* to the USF/MLTF protein (23); interestingly, such motif also fits the binding consensus for the Myc/Max complex (24), whose function in regulating cell proliferation is suggested by several studies (25) [incidentally, the c-*myc* gene domain is amplified in HL-60 cells (26)]. An evolutionary conserved (A+T)-rich region (22) can be envisaged in the proximity of the origin, as is the case for well-defined replication origins of different organisms (27–29).

We have previously shown that the upstream transcript (5000 nt) codes for the human lamin B2 (22), whereas the downstream one (850 nt) contains a 72-amino acid open reading frame with no homology with known proteins except for a Zn-finger motif (to be reported elsewhere); transcription of both genes is highly correlated with cell proliferation (22), in agreement with the correlation between transcription and origin activation well established for several viral and yeast replicons (30, 31).

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