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Utilization of the same DNA replication origin by human cells of different derivation

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ABSTRACT

In the past, a highly sensitive and efficient method was developed to map DNA replication origins in human cells, based on quantitative PCR performed on nascent DNA samples. This method allowed the identification of a replication origin in the myeloid HL-60 cell line, located on chromosome 19 within an ~500 bp segment near the lamin B2 gene [Giacca *et al.* (1994) *Proc. Natl. Acad. Sci. USA*, 91, 7119]. The same procedure has now been further simplified and extended to a variety of other exponentially growing human cells of different histological derivation (three neural, one connectival and one epithelial), with a nearly diploid chromosomal content. In all the six cell lines tested, the origin activity within the lamin B2 gene domain was localized to the same region. Furthermore, the lamin B2 origin was also found to be active in stimulated, but not in quiescent, peripheral blood lymphocytes.

INTRODUCTION

The eukaryotic genome is subdivided, from the point of view of replication, into many tandemly organized units; in animal cells these 'replicons' have typically a size ranging between 5×10^4 and 1.5×10^5 bp (1). Regulation of DNA replication in these organisms is likely to occur by modulation of the rate of activation of specific sequences present in each replicon [origins of DNA replication (*ori*)], in analogy with what is observed in the better defined prokaryotic and viral replicons. The identification of the *ori*s in metazoan cells has proven to be a much harder task than initially predicted and has been marred by repeated failures of the traditional *ori*-searching methods, based primarily on the ability of a given sequence to specifically sustain autonomous replication when inserted into a plasmid (2–4).

During the past few years, new techniques have been devised aimed at mapping the *ori* sites in metazoan chromosomes, based either on the analysis of newly synthesized DNA or on the detection of replication intermediate structures (reviewed in 5–8). The application of these techniques, whose potential drawbacks are in several cases the need for cell synchronization and/or the use of compounds altering cell physiology to enrich for origin-enriched DNA, nevertheless led to the convincing identification of some *ori*s in higher eukaryotes (2,9–16).

Some controversy is still open on whether the *ori*s of animal cells are well defined, relatively short areas of the genome (few tens of bp, like those of prokaryotic organisms and of yeast) or represent instead broad initiation zones with multiple start sites (17–19). However, as the experimental approaches move from the analysis of 2D-gel based autoradiographic patterns to more defined molecular approaches, such as the analysis of the abundance of markers in nascent DNA strands, the evidence for the *ori*s being relatively short and precisely defined regions of the replicons is definitely prevailing.

In our laboratory, over the past years, we have developed a procedure based on the exact quantitation of the abundance of DNA markers in nascent DNA molecules relying on a very precise and sensitive competitive PCR method (20). We have thus been able to identify an origin of human DNA replication within 474 bp (21), located in the intergenic spacer between the end of the lamin B2 gene and the start site of another downstream located and actively transcribed gene (Fig. 1). This replication origin was identified in the human HL-60 cells, a promyelocytic leukemia cell line. By this method, we could also determine that in non-synchronously growing HL-60 cultures, the abundance of the sequence containing the lamin B2 *ori* in the population of short (1000–1500 nt) single-stranded fragments of nascent DNA was approximately one in 30 000. This estimation is consistent with the notion that the analyzed family of short nascent strands corresponds to the family of *ori*s; the latter, in fact, are expected to be present one per replicon, and the number of replicons in human cells has long been indicated to be present in the order of 10^4.

A question then arises whether the same *ori* is utilized also by cell lines of different derivation from the same species. The availability of the competitive PCR mapping technique that we have developed, allows us to address this question in asynchronous cell populations in a relatively simple way. In this report, a further simplification of the PCR mapping procedure is presented, together with the results obtained in five different human cell lines and in primary human peripheral blood lymphocytes.

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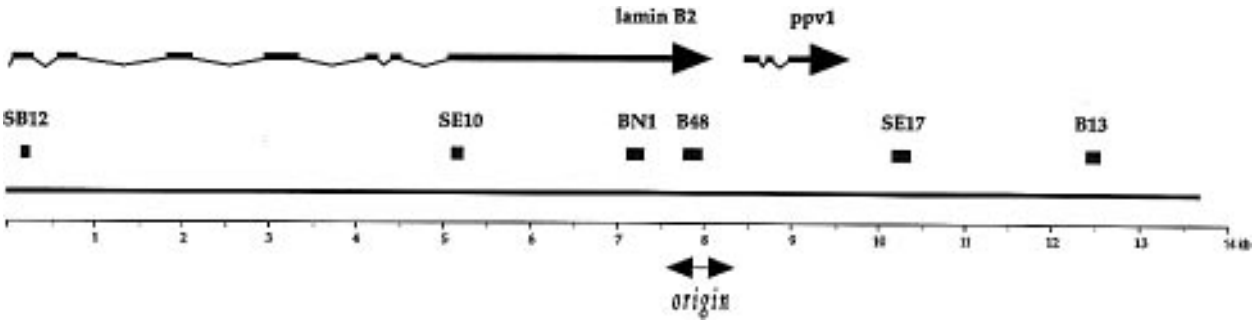

Figure 1. Schematic representation of the human genomic region analyzed in this study. The 13.7 kb region of human chromosome 19 investigated contains the 3′ end of the lamin B2 gene and another tandemly arranged small gene (ppv1). Bold straight portions of the respective gene transcripts shown by arrowed lines represent exons while the vents represent introns. The gray boxes show the locations of the segments amplified by the various primer sets used. The sequence of the core portion of this region (humlambbb) is available in GenBank (accession no. M94363).

MATERIALS AND METHODS

Cell cultures and DNA labeling

HL-60 (myeloid), IMR32 (neuroblastoma) and SKNMC (neuroblastoma) cells were cultured in RPMI 1640 with 15% fetal calf serum; HeLa (epithelial), IMR90 (lung fibroblasts) and SKNBE (neuroblastoma) cells were cultured in Dulbecco's modified Eagle's medium with 12% fetal calf serum. Both media were supplemented with 2 mM glutamine and 50 µg/ml gentamicin.

Peripheral blood mononuclear cells were obtained by density centrifugation of 200 ml peripheral blood from a normal donor over Ficoll-Hypaque (Sigma, St Louis, MI). DNA from resting lymphocytes was obtained from 10^8 non-adherent cells after a 30 min incubation in complete RPMI 1640 medium.

Lymphocytes were activated by incubation in culture medium containing 1 µg/ml phytohemagglutinin (PHA, Sigma) and 10% interleukin-2 (IL-2, Cellular Products Inc., San Diego, CA). Five days after stimulation, DNA was extracted from 10^8 cells.

When appropriate (see below) cells (1×10^8) were pulse-labeled for 10 min at 37°C either with 1 µM (final concentration) [^{3}H]deoxycytidine (21.5 Ci/mmol, Amersham, UK) and 100 µM (final concentration) cold 5′-bromodeoxyuridine (BrdUrd; Boehringer Mannheim GmbH, Mannheim, Germany), as described (21) or with 1 µM (final concentration) [^{3}H]thymidine (15.1 Ci/mmol, Amersham).

Extraction and purification of newly synthesized DNA

Total DNA was extracted from exponentially growing, asynchronous cell cultures by standard procedures (22), denatured by a 10 min incubation in boiling water, and size-separated (300 µg per gradient) on 35 ml of 5–30% neutral sucrose gradients for 20 h at 26 000 r.p.m. in a Beckman SW28 rotor at 20°C. In parallel, a reference tube with a double-stranded size marker DNA (containing four different sized DNA fragments in the range of 500–5000 bp) was also run on an identical gradient. Fractions of 1 ml each were collected and those from the gradient with the markers were run on 1% agarose gel at 30 V overnight. Using the separation pattern of the marker DNA on the gel as a sedimentation velocity reference, the fractions containing ssDNA of ~1 kb were selected in each case and dialyzed against Tris–EDTA (0.5 M Tris pH 8; 0.01 M EDTA) for at least 8 hours. These fractions were then used to quantify ssDNA by competitive PCR using different sets of primers in the lamin B2 region. Only in the case of SKNBE cells, a 10 min incubation of the cells in 100 µM BrdUrd and 1 µM [^{3}H]deoxycytidine in complete RPMI 1640 medium was performed prior to DNA extraction, and the size-selected fractions were purified by immunoaffinity chromatography using anti-BrdUrd antibodies (23) before proceeding to quantitative PCR (this is exactly the same procedure as reported in 21; Fig. 2A). The variations to this standard procedure (i.e. omission of the immunoaffinity step, omission of the BrdUrd labeling step, labeling with [^{3}H]thymidine 1 µM, etc.) are reported in Table 1. Analysis of all samples in Figure 4 was according to the simplified protocol outlined in Figure 2B.

PCR amplification and competitor construction

The primers used for competitive PCR experiments in the lamin B2 genomic area (primer sets B13, B48, BN1, SB12, SE10 and SE17) and within the β-globin gene (primer set PCO) have already been described (21). These primers amplify segments of 100–300 bp; their position in the lamin B2 genomic area is shown in Figure 1. For each primer set, competitor DNA segments were constructed carrying the corresponding genomic sequence with the addition of 20 extra nucleotides in the middle, to allow gel electrophoretic resolution of the amplification products. The procedure for competitor construction was the same as described by Diviacco *et al.* (20).

Competitive PCR experiments were carried out by the addition of scalar quantities of competitor to a fixed amount of each nascent DNA preparation and subsequent amplification with the appropriate primer sets. This procedure and the individual PCR profiles have already been described (21).

RESULTS

A simplified origin mapping procedure

The original method for DNA replication origin mapping (21), as described in Figure 2 protocol A, consisted of: (i) bromodeoxyuridine (BrdUrd) and [^{3}H]deoxycytidine pulse-labeling of the newly synthesized DNA; (ii) DNA extraction and size fractionation of short nascent DNA strands (~1 kb); (iii) further purification of newly synthesized, BrdUrd-substituted DNA by immunoaffinity chromatography using anti-BrdUrd antibody coupled to Sepharose beads; and (iv) quantitation of DNA segment abundance in the purified sample by competitive PCR. By this procedure, nascent DNA (i.e. short DNA stretches

emanating from the origin) is firstly purified from total newly synthesized DNA (deriving from all regions in the genome, the cells being unsynchronized) according to its size and then further enriched from the bulk unreplicated genomic DNA by affinity chromatography. Since the origin mapping procedure by fragment abundance detection relies in principle on size selection only, we attempted a simplification of this procedure by omitting the affinity chromatography step. Accordingly, nascent DNA strands of ~1 kb in size isolated from exponentially growing asynchronous cells of the neuroblastoma cell line SKNBE were directly submitted to quantitative PCR analysis (Fig. 2B) using six sets of primers spanning the region of interest (Fig. 1) and one control primer set (primers PCO3 and PCO4), which detects an unrelated region in the β-globin gene. As shown in Table 1 (rows A and B), the omission of the BrdUrd-purification step did not give any appreciable differences neither in the detectability of DNA segments by competitive PCR nor in the relative abundances of the markers. On the contrary, omission of this step, besides simplifying the procedure, also avoids the introduction of a possible bias in fragment selection on the anti-BrdUrd affinity column and reduces the possibility of DNA fragmentation by photodamage.

Given the above reported results, BrdUrd labeling was no longer utilized, and the simplified procedure of Figure 2 protocol B was consistently followed. Line C of Table 1 reports the results obtained by the application of this procedure to the HL-60 cell line, where the lamin B2 origin had originally been mapped within ~500 bp encompassing primer set B48 (21). Comparison of the marker abundance distribution in this cell line with that obtained in SKNBE cells indicates that also the latter utilizes the lamin B2 *ori*.

We have previously shown, by utilizing the BrdUrd procedure in asynchronous cells, that the lamin B2 *ori* was present with a frequency of approximately one molecule out of 30 000 nascent DNA strands (21). This proportion is analogous to what one would expect if the short nascent DNA molecules were a faithful representation of the population of all replicon origins. The same measurements were now repeated in HL-60 cells by utilizing the simplified procedure involving only fractionation by size, with the addition of a ^{3}H label to allow the absolute quantitative determination of the total number of molecules present in the sample (Table 1, row D*). By this procedure, we could estimate that the abundance of lamin B2 origin-containing fragments was of one molecule per 36 000 nascent DNA fragments. The value is significantly close to the previously reported one, thus further confirming the validity of the simplified procedure.

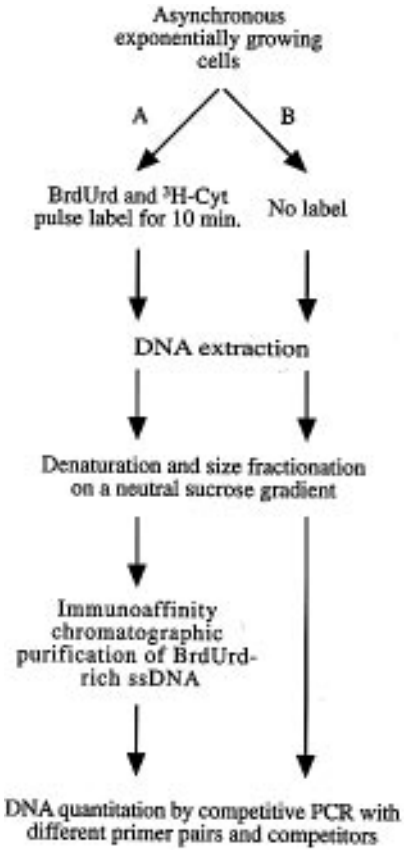

Figure 2. Flow chart diagram showing the original (**A**) and the simplified (**B**) procedures for preparing newly replicated DNA fractions for origin mapping studies.

Table 1. Comparison of the complete and simplified procedures for origin mapping by quantitation of DNA segment abundance in samples of nascent DNA

Cell line	Method used	Markers						
		SB12	SE10	BN1	B48	SE17	B13	PCO
		Relative abundance (arbitrary scale)						
SKNBE	A	<500	300	900	1600	400	120	200
	B	450	300	900	1200	ND	120	200
HL60	C	400	600	1200	3000	850	400	500
	D	400	500	1200	3000	900	400	500
		Absolute abundance (number of primer-specific molecules/10^6 total ssDNA molecules)						
	D*	4.8	7.2	14.4	36.1	10.2	4.8	6.0

A: complete procedure as shown in Figure 2 protocol A and explained under Materials and Methods.
B: same as A but with the omission of the immunoaffinity chromatography step (Fig. 2, protocol B).
C: same as B but without any labeling of cells (BrdUrd or radioactivity).
D: same as C with a 10 min incubation of the cells with [^{3}H]thymidine prior to DNA extraction.
D*: same as D, but the results are expressed as absolute amounts, i.e., number of primer-specific molecules/10^6 total ssDNA molecules.

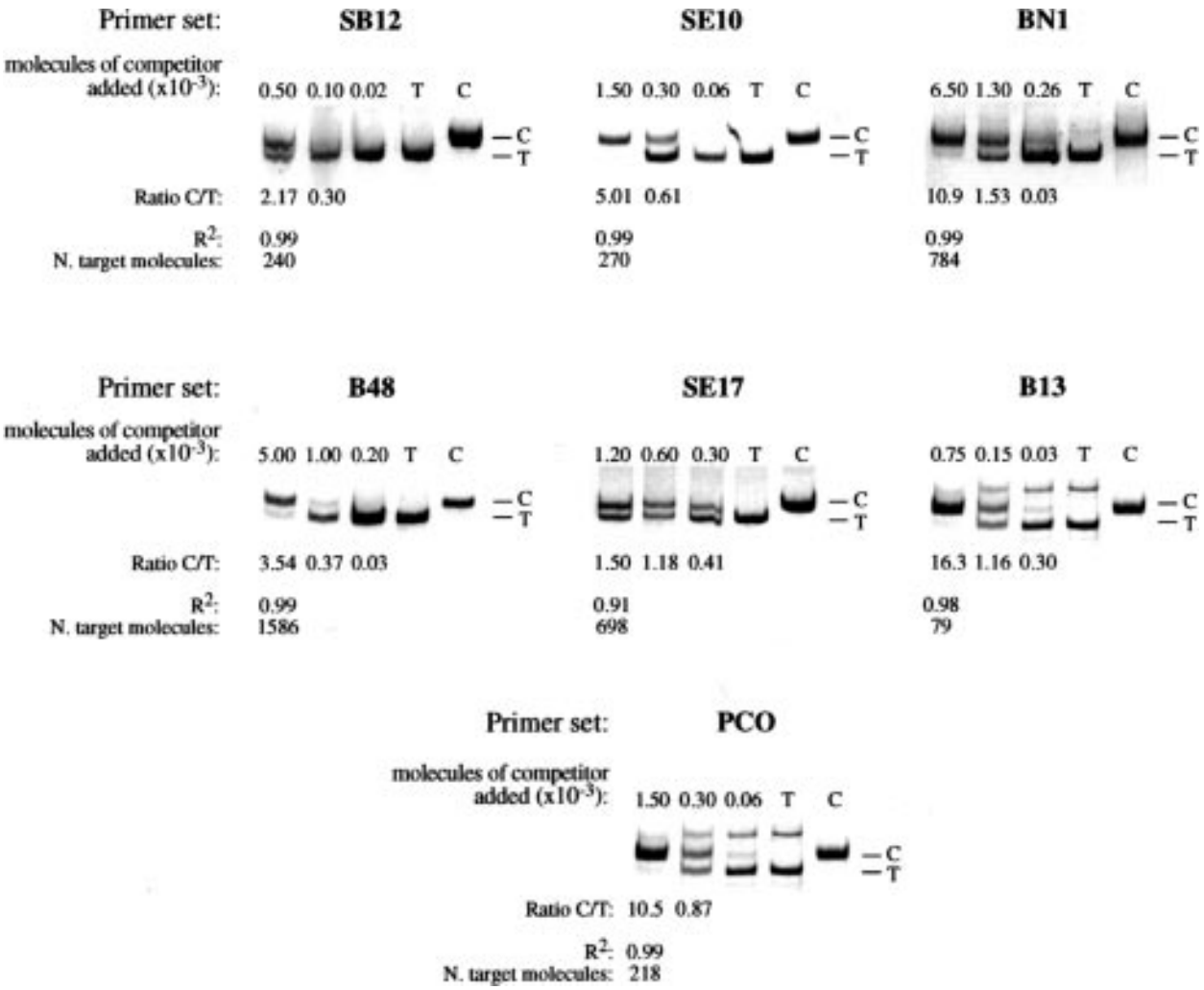

Figure 3. Results of competitive PCR experiments for the quantification of the indicated seven genomic segments in a newly replicated DNA sample (~1 kb average size) from asynchronously growing IMR90 cells. For each primer set, a fixed amount of newly synthesized DNA was co-amplified with the amount of competitor fragment indicated at the top of each gel. PCR products were resolved by polyacrylamide gel electrophoresis, stained with ethidium bromide and photographed. The intensity of the bands corresponding to the PCR products for the competitor (C) and the target genomic (T) DNAs was assessed by densitometric scanning of each lane. The ratio between the two PCR products (reported at the bottom of the gels for each lane in which quantification of both species could be performed) is linearly correlated with the quantity of competitor added to the reaction; the correlation coefficients are reported for each gel. From the equation of the line fitting the experimental points (20), the number of target molecules was evaluated and is reported at the bottom of each panel.

The lamin B2 origin in other human cell lines

The simplified procedure described in the previous paragraph was applied to different cell lines. Figure 3 shows the polyacrylamide gels obtained after a competitive PCR experiment performed with a nascent DNA sample obtained from the human lung fibroblasts cell line IMR90. A fixed volume of sample DNA was amplified by seven primer sets (SB12, SE10, BN1, B48, SE17, B13 in the lamin B2 genomic region, and PCO in the β-globin gene) in the presence of different quantities of the respective competitors, as indicated at the top of each gel. According to the principles of competitive PCR (20), the ratio between the competitor and genomic template amplification products (shown at the bottom of each gel) is linearly related to the number of competitor molecules initially added to the reaction. Since the latter amount is known, the concentration of each investigated genomic segment in the sample could be calculated easily from the interpolation of the regression line.

For each genomic segment to be quantified, competitive PCR experiments were firstly carried out with 10-fold scalar dilutions of the corresponding competitor, to roughly estimate the target concentration; more precise quantification was subsequently obtained by using 5- or 2-fold competitor dilutions, as shown in Figure 3. Similarly, precise quantification of each competitor was independently obtained by competitive PCR experiments with a fixed amount of total human genomic DNA.

The results obtained for the IMR90 cell line are graphically shown on the upper left panel of Figure 4. The other panels of Figure 4 show the results of similar experiments performed on cell lines of different histological derivation. These include the IMR32, SKNBE and SKNMC neuroblastoma, the HL-60 myeloid and the HeLa epithelial cell lines. In all these cells, a clear

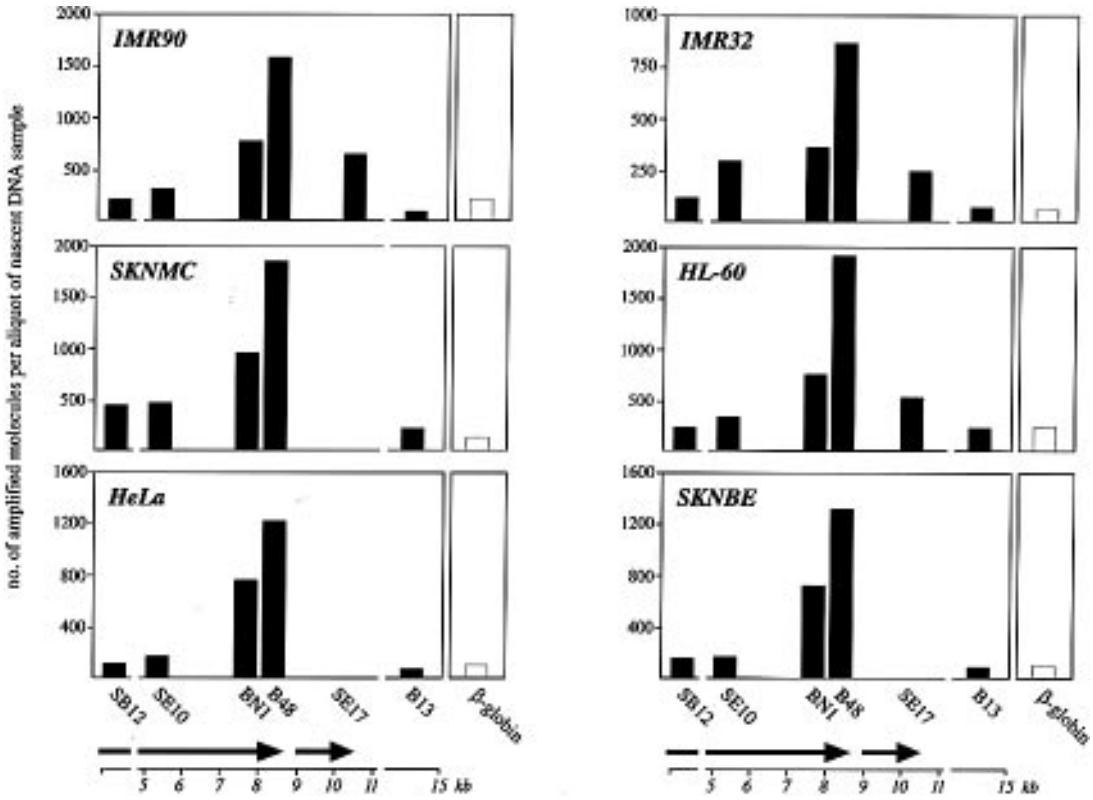

Figure 4. Graphical representation of the results obtained by the quantification of different segments in the lamin B2 gene domain by competitive PCR of nascent DNA samples from the indicated cell lines. PCR quantitation results are given in terms of an arbitrary value, i.e. number of primer-specific molecules amplified in a fixed volume of the ssDNA pool tested for each line. Quantification of the β-globin gene copy number was obtained by the PCO primer set.

enrichment could be detected for the genomic fragment corresponding to the B48 marker—the one corresponding to the lamin B2 *ori* region (21), scoring 6–10-fold higher than the markers localized >5 kb apart on each side (SB12 and B13), and those of the β-globin gene control region.

This distribution of DNA segment abundance in samples of nascent DNA clearly suggests that the lamin B2 *ori* is active in all the analyzed cell lines.

Absence of origin activity in quiescent primary cells

In addition to the results reported above, which were obtained in established cell lines that replicate at a continuous rate in the absence of inhibitory treatments, the lamin B2 *ori* activity was studied in peripheral blood lymphocytes from a normal individual. These primary cells are largely quiescent in the absence of stimulation. When size-selected single-stranded DNA was analyzed from these cells, no *ori* activity could be detected in the lamin B2 region, as expected (Fig. 5A). However, when the PCR mapping experiment was performed on nascent DNA samples extracted from the same lymphocytes at 5 days after stimulation (obtained by the addition of phytohemoagglutinin and interleukin-2), a clear evidence of activation of the lamin B2 *ori* could be observed, peaking at the same region as in the cell lines. This observation, besides providing an important control to the results obtained in the established cell lines, represents the first observation of origin usage in primary human cells.

DISCUSSION

The use of BrdUrd pulse-labeling of newly replicated DNA and of the anti-BrdUrd antibodies in the process of template DNA preparation for origin-detection experiments was originally introduced (23,24) with the aim of obtaining a high signal-to-noise ratio. We show here that the method can be further simplified and that the use of BrdUrd and immunoaffinity chromatography can be omitted. This result can be attributed to careful size selection of the nascent DNA strand pool to be used as well as to the reduction in the background (i.e. pool contamination by aspecifically broken DNA) resulting from the elimination of the BrdUrd-substituted areas in the DNA which are prone to breakage. The value of ~3×10^4 different *ori*s inferred by our data in human cells agrees with the occurrence of an origin every ~100 kb, as originally suggested by the fiber autoradiographic studies on the mammalian DNA replication fork movement (25). This new simplified procedure for origin mapping now provides a simple tool for the study of the process of DNA replication in single copy domains of untreated, asynchronously growing cells. Its extensive application will be suitable for the identification of novel origins in mammalian cell DNA and will assist in the understanding of the functional organization of the genome.

As far as the lamin B2 *ori* in the chromosome 19p13.3 location is concerned, the results reported here show that this origin is used to initiate DNA replication in a variety of human cells. Of particular interest is the observation that this *ori* is activated also

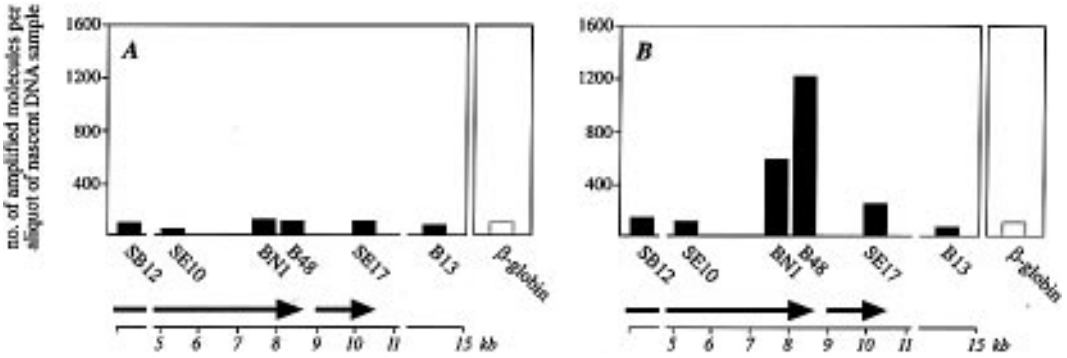

Figure 5. Quantitative PCR results for the indicated primer sets in 1 kb DNA fragment pools obtained from quiescent (**A**) and proliferating (**B**) human peripheral blood lymphocytes from a normal donor.

in primary peripheral blood lymphocytes, providing further support to the physiological significance of the observations obtained in the established cell lines. The absence of origin activity in unstimulated, quiescent lymphocytes goes in parallel to our previously reported observation where also HL-60 cells, when differentiated *in vitro* to a non-proliferating state, gave no evidence of lamin B2 *ori* activity (21). Once again, these observations reinforce the validity of the developed method for origin identification.

The universal use of this origin in all the analyzed cells is not surprising. Given the close relationship between transcriptional activity and initiation of DNA replication (5), possibly related to the accessibility of chromosomal domains to proteins involved in both processes, the observation that this origin lies in a constitutively expressed gene domain, coding for a house-keeping protein (26) predicts origin usage by all cells. Further support to this notion is the observation that, in synchronized cells, this *ori* fires within the first minutes of S phase (27).

The observation that the same precise and rather narrowly defined region of the chromosome works as an *ori* in very different cell types gives still more weight to the conception that *ori*s in higher organisms are constituted by *cis*-acting sites of spatially and temporally programmed interactions with *trans*-acting specific protein factors. A study of the nature of the proteins involved in the definition and activation of the human lamin B2 *ori* has been initiated (28).

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