

Cell cycle modulation of protein–DNA interactions at a human replication origin

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We followed the variations of protein–DNA interactions occurring *in vivo* over the early firing replication origin located near the human lamin B2 gene, in IMR-90 cells synchronized in different moments of the cell cycle. In G₀ phase cells no protection is present; as the cells progress in G₁ phase an extended footprint covering over 100 bp appears, particularly marked at the G₁/S border. As the cells enter S phase the protection shrinks to 70 bp and remains unchanged throughout this phase. In mitosis the protection totally disappears, only to reappear in its extended form as the cells move into the next G₁. These variations are reminiscent of those corresponding to the formation of the pre- and post-replicative complexes described in yeast and *Xenopus* cells.

Keywords: DNA replication origin/human genome/*in vivo* footprinting/origin-binding proteins/pre-replication complex

Introduction

The eukaryotic genome consists of many tandemly organized replicons (in the human species, ~30 000, assuming an average length of 100 kb) (Huberman and Riggs, 1968; Falaschi and Giacca, 1994) that are each duplicated, once and only once per cell cycle, at different times of the S phase. The study of eukaryotic microorganisms has allowed the identification within their replicons of precise origins of bidirectional replication specified in *Saccharomyces cerevisiae* by a consensus sequence as short as 11 bp (Marahrens and Stillman, 1992; Rao *et al.*, 1994; Theis and Newlon, 1994); furthermore, these origins were shown to behave as dynamic structures on which an array of specific proteins assembles and disassembles during each round of cell division. In particular, *in vivo* footprinting of a *S.cerevisiae* origin showed that at least two different protein–DNA complexes, pre- and post-replicative, occupy the origin at different moments of the cell cycle (Diffley *et al.*, 1994); also, *in vitro* studies of *Xenopus laevis* extracts support a model of temporally ordered assembly of specific factors at the origins as replication proceeds (Blow, 1996; Coleman *et al.*, 1996; Romanowski *et al.*,

1996; Rowles *et al.*, 1996). In more detail, origin selection in *S.cerevisiae* is brought about by a complex of six proteins called the origin recognition complex (ORC) that marks the origin and remains bound to it throughout the cell cycle (Bell and Stillman, 1992; Diffley *et al.*, 1994; Rowley *et al.*, 1995; Aparicio *et al.*, 1997; Tanaka *et al.*, 1997). More recent developments are suggestive of mechanisms whereby the activated origin cannot be activated again until the next S phase and the origin activation event is linked to the progress of the cell cycle. It appears that an ordered assembly of a multiprotein complex occurs during the cell cycle; in particular, in *S.cerevisiae*, the origin-bound ORC, at some moment before the G₁–S transition, first binds the protein encoded by the *Cdc6* gene, and immediately afterwards the six mini-chromosome maintenance (MCM) proteins (Liang *et al.*, 1995; Cocker *et al.*, 1996; Lei *et al.*, 1996; Santocanale and Diffley, 1996; Donovan *et al.*, 1997; Tanaka *et al.*, 1997); this process also requires the action of a protein kinase encoded by the *Cdc7* gene (Dowell *et al.*, 1994). Thus, a pre-replicative complex (pre-RC) is formed which binds across the origin at a significantly longer sequence than does the ORC and, at the appropriate moment of S, causes the firing of two bidirectional replication forks; the destruction of *Cdc6p* and the detachment of the MCM proteins from the origin cause the disassembly of the pre-RC, leaving only the ORC to mark the origin (Diffley, 1996; Stillman, 1996). It is not until completion of mitosis that a new pre-RC is allowed to form; this event is concomitant with the synthesis of new *Cdc6p* and the inactivation of the mitotic B-type cyclin/*Cdc28* kinase complex, which plays an inhibitory role for pre-RC formation during G₂ and M phase (Schwob *et al.*, 1994; Dahmann *et al.*, 1995).

The mechanism summarized here, drawn mainly from the work performed in *S.cerevisiae*, has found corroboration in the results obtained in other eukaryotes, whether microorganisms (e.g. *Schizosaccharomyces pombe*) or metazoans; in particular, for the latter, both in *X.laevis* and in human cells the putative counterparts of the ORC, MCM and *Cdc6* proteins have been identified and, in most cases, their genes have been cloned and sequenced (Gavin *et al.*, 1995; Wang, and Li, 1995; Chong *et al.*, 1996; Rowles and Blow, 1997; Williams *et al.*, 1997). However, somewhat paradoxically, in metazoans the identification of precise replication origins is much less satisfactory and still controversial, a situation that hampers the possible identification of specific binding to them of the elements of the ORC and pre-RC. In CHO cells, in the area near the *DHFR* gene, an origin of replication has been reported to be located within a few hundred basepairs by some groups, including ours (Burhans *et al.*, 1990; Pelizon *et al.*, 1996) and to be relatively delocalized, within as many as 50 kb, by others (Vaughn *et al.*, 1990). In *X.laevis*, during the first part of embryonic development

(the first 12 cell divisions until midblastula), any sequence seems to work as an origin, but afterwards the origins are restricted to limited sequences (Hyrien *et al.*, 1995). In the fly, *Sciara coprophila*, the same DNA sequence, when undergoing the multiple rounds of replication required for the localized gene amplification involved in the formation of a differentiating larval puff, initiates replication preferentially within 1 kb while, when undergoing the standard endoreduplication occurring in salivary glands, it provided evidence of diffuse initiation events in an area of as many as 7 kb (Liang *et al.*, 1993).

In recent years we have developed a very sensitive origin mapping technique that allowed us to locate a replication origin within 500 bp in a region of the G-negative band p13.3 of human chromosome 19 (Giacca *et al.*, 1994); the origin area overlaps the 3' non-coding end of the gene for lamin B2 and the neighbouring half of the ~600 bp-long spacer preceding another (still uncharacterized) gene, named *ppv1*. This origin is active in a variety of human cell lines of different derivation: the myeloid HL-60 cell line, in which the initial identification of the origin was performed; peripheral lymphocytes; epithelial cells (HeLa); neuroblastoma; and fibroblast cell lines (Kumar *et al.*, 1996). *In vivo* footprinting analysis performed by the ligation-mediated PCR technique over and around the origin area showed a number of footprints indicative of specific protein–DNA interactions (Dimitrova *et al.*, 1996). Some of these correspond to sequences of well-known transcription factors (namely, the sequence recognized by the basic helix–loop–helix family of proteins, and three adjacent sequences for the Sp1, NRF-1 and UBF factors, respectively); judging from their position over the promoter area of the *ppv1* gene and from their persistence in terminally differentiated cells (where replication is blocked whereas the gene is still transcribed), we concluded that they are most probably related to the *ppv1* gene transcription. Conversely, further upstream of the *ppv1* promoter area, and partially overlapping the end of the sequence complementary to the mRNA of the lamin B2 gene, a very prominent, >70 bp footprint was observed, having a sequence that bears no similarity to the sequences bound by known transcription or replication factors. This protection concerned mainly (although not exclusively) the strand complementary to the transcript and showed a bipartite structure, with a non-protected guanosine residue near the middle. When the HL-60 cells were differentiated in culture and no longer proliferating, the >70 bp footprint completely disappeared.

This observed proliferation-correlated binding is clearly reminiscent of that of the yeast ORC and prompted us to investigate further for possible variations in the different phases of the cell cycle. We report here the results of this investigation.

Results

The analysis of protein–DNA interactions occurring *in vivo* at the origin of replication located near the lamin B2 gene was performed by genomic footprinting in human cell lines of different histological derivation and in different phases of the cell cycle. Genomic footprinting was obtained by *in vivo* DNA methylation using dimethylsulfate (DMS) followed by the analysis of modified nucleotides

using ligation-mediated PCR. Five different primer sets were used for the analysis, the localization of which is reported schematically at the top of each footprinting figure. Each primer set is composed of three overlapping oligonucleotides; primer sets G and D correspond to sequences of the lamin B2-coding (upper) strand, thus detecting protein–DNA interactions occurring on the lower strand, while primer sets E, C and A correspond to sequences of the non-coding (lower) strand in the promoter region of the *ppv1* gene. The combined use of these primers allowed the analysis of ~900 bp on both strands (nucleotides 3473–4385 of GenBank file humlambbb). The origin of DNA replication had been located within nucleotides 3880–4107 by competitive PCR mapping experiments (Giacca *et al.*, 1994).

Protein–DNA interactions at the lamin B2 origin in different cell lines

We have already shown that in HL-60 cells the central portion of the analysed area is involved in a large proliferation-dependent footprint which completely disappears in terminally differentiated cells (Dimitrova *et al.*, 1996). The same genomic region (called the OBP region, since it appears to be protected by Origin Binding Proteins) was then analysed by the use of primer set D to assess whether it was protected also in other cell types which have previously been shown to use the lamin B2 origin (Kumar *et al.*, 1996). The analysed cell lines include IMR-90 primary lung fibroblasts, Jurkat T-lymphoblastoid cells, HeLa epithelial cells and peripheral blood lymphocytes from a normal donor; the results of this analysis are shown in Figure 1B. A large footprint is evident in all these cells [Figure 1B, compare lanes 1, 4 and 6 (naked DNA) with lanes 3 (asynchronously growing IMR-90 cells), 5 (Jurkat cells), 7 (HeLa cells) and 9 (phytohaemagglutinin-activated peripheral blood lymphocytes)]. The boundaries of this footprint are nucleotides 3991 and 3912; the protected region contains a prominent non-protected guanine at position 3964. We will refer to this 78 nt footprint as the OBP box, with analogy to the same protection already detected in HL-60 cells (Dimitrova *et al.*, 1996).

The protection described appears exclusively in proliferating cells. Both in IMR-90 fibroblasts which were submitted to serum starvation and entered G₀ (as detected by cytofluorimetry; see next paragraph; Figure 1B, lane 2) and in peripheral blood lymphocytes in the absence of stimulation (Figure 1B, lane 8), the pattern obtained by DMS methylation is virtually indistinguishable from the pattern of naked DNA treated with DMS *in vitro*, in agreement with the previous observation in non-proliferating HL-60 cells (Dimitrova *et al.*, 1996). In addition, it can be observed that the protection over the OBP box in activated peripheral blood lymphocytes is not as complete as in the cell lines, in agreement with the notion that the B cells which are present in the lymphocyte population are not activated by phytohaemagglutinin.

Analysis of protein–DNA interactions in different phases of the cell cycle: *ppv1* gene promoter area

The study of the modulation of protein–DNA interactions in the lamin B2 origin area during the cell cycle was performed in human IMR-90 diploid fibroblasts that undergo proliferation arrest upon serum starvation. The

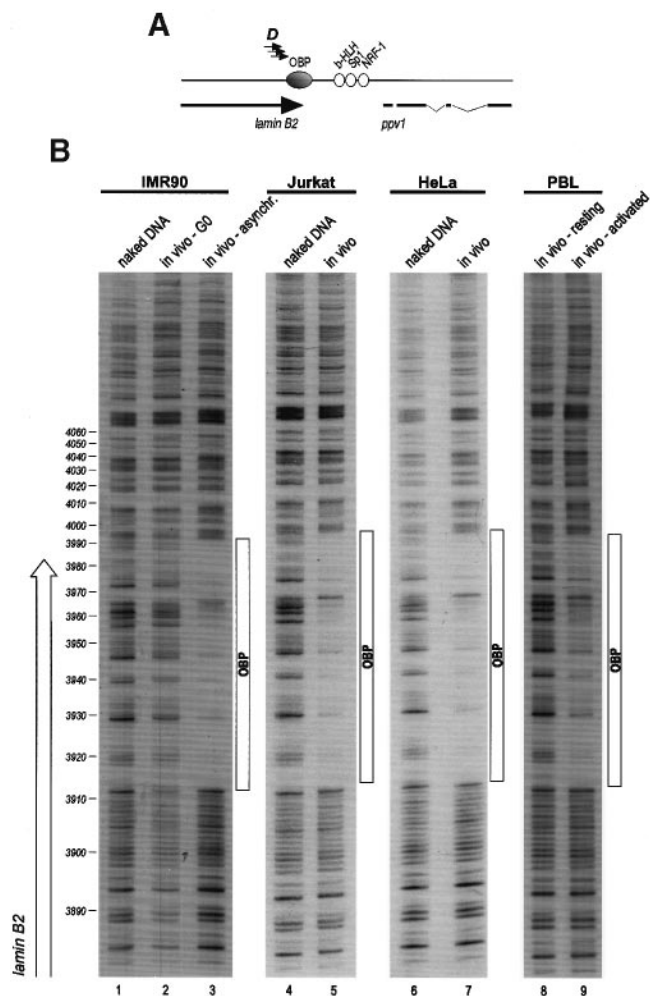


Fig. 1. Proliferation-correlated protection of the OBP region in different cell lines utilizing the lamin B2 origin. (A) Schematic representation of the DNA–protein interactions at the lamin B2 origin. The cluster of arrows indicates the localization and orientation of the primer set D used to reveal lower strand sequences from nucleotides 3850 to 4090. (B) Human cells of different derivation were subjected to genomic footprinting either in G_0 (lanes 2 and 8) or during exponential growth (lanes 3, 5, 7 and 9). Lanes 1, 4 and 6 are naked DNA controls. The sequence corresponding to the OBP site is indicated by open boxes. The numbers on the left side refer to the file humlambbb of GenBank.

synchronization procedure is outlined in Figure 2A. IMR-90 cells were cultured for 3–4 days in the absence of serum; under these conditions, only a peak at the $2n$ position is visible by cytofluorimetric analysis (G_0 sample in Figure 2B). Serum addition causes re-entry of cells into the cell cycle (as judged by thymidine incorporation experiments; not shown) and subsequent re-entry into mitosis. Thus, cells collected at 2, 4 and 6 h after serum addition are considered as G_1 cells, and their DNA profile shows the same $2n$ content as G_0 cells (Figure 2B). Cells in which serum stimulation was carried out for 24 h in aphidicolin-containing medium (aphidicolin is an inhibitor of replicative DNA polymerases) accumulated at the G_1/S border; removal of aphidicolin and subsequent incubation for 5 h provided cells, 80% of which were in S phase (S profile in Figure 2B). After additional 5 h and 30 min incubations, the cells began to enter mitosis (M profile in Figure 2B). The population of mitotic cells was

enriched by collecting non-adherent cells, and either analysed directly or replated to obtain a population of cells which had re-entered the subsequent G_1 after an additional 8 h incubation. Samples of cells harvested at different points in the synchronization were tested for specific protection by *in vivo* footprinting with primer sets covering the origin region.

The results obtained with primer set A are shown in Figure 3. This primer set is localized in the promoter region of the *ppv1* gene, downstream of the binding sites for transcription factors NRF-1, Sp1 and for proteins of the basic-helix–loop–helix (b-HLH) family (Figure 3A). We have evidence that USF and NRF-1 do indeed bind to these sequences *in vitro* (Csordás Toth *et al.*, 1993; unpublished data). The genomic footprints corresponding to the binding sites for these factors are clearly evident in the *in vivo*-treated samples shown in Figure 3B (lanes 4–9). Protection of these sequences is recognizable in cells in G_0 , G_1 , G_1/S border and S phase, without any appreciable differences in intensity or pattern. This agrees with our previous observation that *ppv1* is also transcribed at appreciable levels in non-proliferating cells (Dimitrova *et al.*, 1996).

In contrast to HL-60 cells, the additional footprint immediately downstream of the NRF-1 site (a sequence homologous to the binding site of the RNA polymerase I transcription factor UBF; Dimitrova *et al.*, 1996) is not visible in the IMR-90 cell line.

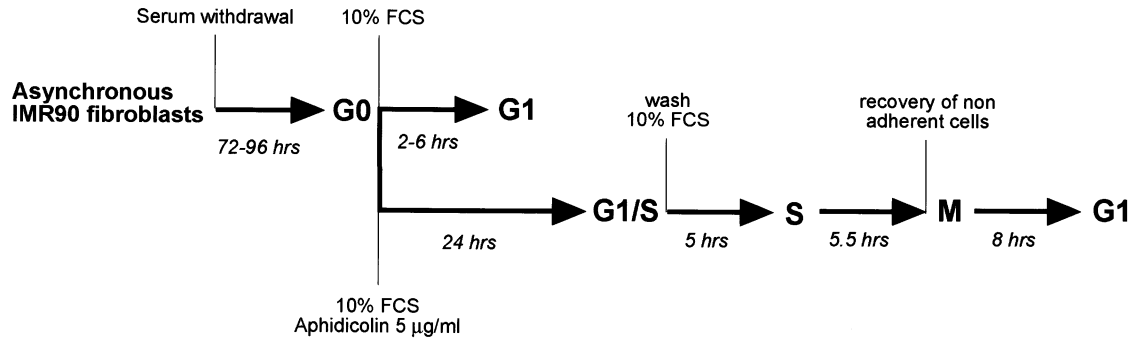
Interestingly, when the cells move into mitosis and the chromosomes are tightly packaged, all the detected footprinting are no longer visible (Figure 3B, lane 10), suggesting that these transcription factors have been ‘squeezed off’ the DNA.

Analysis of protein–DNA interactions in different phases of the cell cycle: intergenic and origin area

The analysis of the region upstream of the *ppv1* promoter was performed by using primer sets C and E, and is presented in Figure 4. In the region analysed by primer set C, there are no appreciable protections downstream of nucleotide 4000. Conversely, as we move upstream, both primer sets begin detecting footprints at position 3891 in G_1 cells (Figure 4B, lane 4; Figure 4C, lane 3). This G_1 footprint extends to nucleotide 3940 (box A' in Figure 4; see Discussion for labelling of the different protected regions). Another protection (box B') is also visible in G_1 cells, from nucleotides 3954–3995. Remarkable changes are observed in the footprinting pattern of this region when S cells are compared with G_1 cells. The B' protection completely disappears in S cells, while the A' protection is markedly reduced (Figure 4, box A1'; lanes 4 in both panels). It should be observed that the exact boundaries of box A1' cannot be clearly determined by DMS treatment, since this region contains a single G nucleotide on the upper strand. In striking contrast to the results obtained for the *ppv1* promoter region, all the protections in this region are absent in G_0 cells (Figure 4B, lane 5). Additionally, it is of interest that in this region no protections are detectable in cells collected during the M phase (Figure 4A and B, lane 2).

The lower strand of the origin area was analysed in G_1 cells by primer sets G and D (Figure 5). A very large footprint is detectable from nucleotides 3913–4006 (Figure

A



B

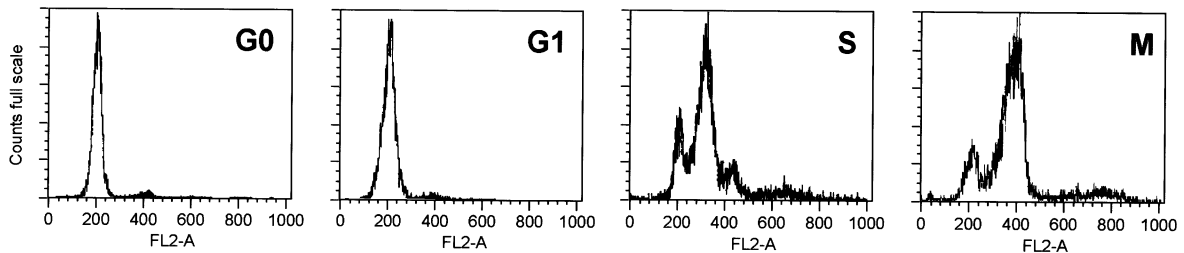


Fig. 2. Synchronization of human IMR-90 fibroblasts. (A) Schematic representation of the synchronization strategy. (B) Flow cytometric analysis of propidium iodide-stained IMR-90 cell populations collected at different stages of the cell cycle.

5, box A), flanked by a short footprint from nucleotides 3891–3910 (Figure 5, box B). This extended protected area (116 nt in total) is flanked at the 3' side where five adenosines (which are less reactive to DMS) also appear to be protected (empty arrowheads in Figure 5C) and by a region at the 5' side where two strong hypersensitive sites are located (filled arrowheads in Figure 5C). The latter region is better resolved in the longer gel run presented in Figure 5D.

Contrary to the pattern observed in G_1 cells, and with remarkable similarity to the situation reported for the complementary strand, G_0 cells show an *in vivo* DMS sensitivity pattern which is indistinguishable from that of naked DNA (Figure 5B, lane 2; Figure 5C, lane 3). The protection over this region appears to increase progressively during transit of the cells in G_1 , since the footprinting pattern is only weakly appreciable at 2 h following serum addition (Figure 5C, lane 4) and becomes virtually complete after 5 h. These data, together with the absence of protection in G_0 cells, are consistent with the progressive assembly of a large protein complex while cells move through G_1 .

In S cells (Figure 5B, lane 4; Figure 5C, lane 9), the protection pattern over the region shows remarkable changes; the B box is no longer protected, the large A box becomes split into two smaller A1 and A2 boxes, separated by a non-protected guanosine at position 3964, and the flanking-protected, hypersensitive adenosine stretch and guanosine sites are no longer evident, contrary to the G_1 pattern. These data are consistent with the partial disassembly of the large protein complex present in G_1 following activation of the origin, and the permanence of a smaller complex marking the already utilized initiation

site. This inference is further strengthened by the observation that shrinking of the footprint from the G_1 to the S pattern occurs even if fork advancement is forbidden; Figure 5C, lane 14, shows the pattern obtained with cells which were accumulated at the G_1/S border by a 24 h incubation with 5 $\mu\text{g/ml}$ of aphidicolin, a drug which inhibits chain advancement but not initiation. Also, in these conditions the protection observed is that typical of the S cells, that is, of cells in which the initiation event at the investigated origin has already taken place.

As cells progress into M, again similar to that detected for all the protections, the DMS sensitivity pattern becomes indistinguishable from that of naked DNA (Figure 5B, lane 3; Figure 5C, lane 10). Finally, when the same synchronized cells are allowed to undergo another cell cycle and are analysed during the subsequent G_1 phase, the pattern detected shows remarkable identity to that obtained in the previously analysed G_1 phase, with the appearance of large A and B boxes, and of the same flanking alterations in the lower strand (Figure 5C, lane 12).

Discussion

An overall view and analysis of the footprinting patterns over the origin area in the G_1 and S phases is represented schematically for both strands in Figure 6. The large protection observed in G_1 on the lower strand, which appears divided in two unequal portions, is labelled as A and B boxes, respectively; the matching footprints observed on the upper strand in the same region are accordingly labelled A' and B' boxes; the bipartite footprint observed in the lower strand in S, which is within the larger A box, is divided in two boxes labelled A1 and

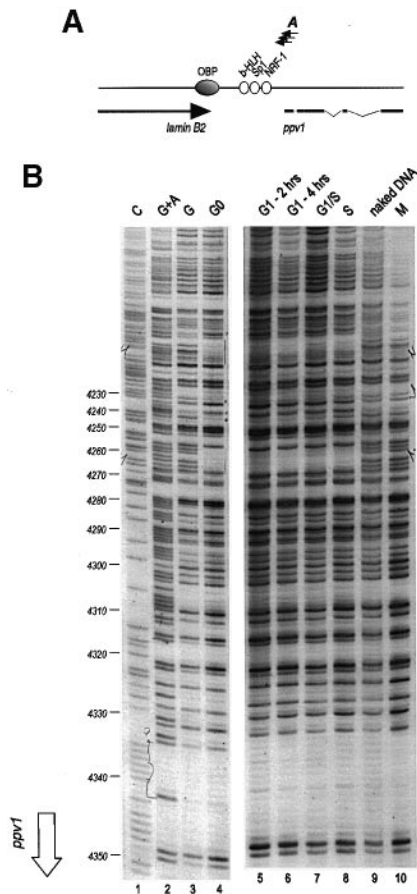


Fig. 3. DNA–protein interactions on the upper strand in the region of the promoter of the *ppv1* gene. (A) Primer set A was used to visualize sequences on the upper strand from nucleotides 4350 to 4200. (B) Human fibroblasts IMR-90 were synchronized at the indicated phase of cell cycle and then subjected to genomic footprinting as described in materials and methods. Lanes 1–3 are Maxam–Gilbert sequencing reactions. The consensus binding sites for transcription factors NRF1, Sp1 and b-HLH are indicated by open boxes. The numbers on the left refer to the file humlambbb of GenBank.

A2; correspondingly, the smaller footprint present in the opposite upper strand in S is labelled A1'.

In the same scheme, the localization of the OBP protection detectable on the lower, non-coding strand in asynchronous cell populations (Dimitrova *et al.*, 1996) is also indicated. This footprint corresponds remarkably well to the protection observed in S-phase cells. Additionally, we indicate the position of the two primer pairs (B48DXL and B48SXL) which were shown, in competitive PCR mapping experiments, to include the origin area (Giacca *et al.*, 1994). As is evident from Figure 6, the region encompassed by these primers corresponds precisely to the location of the above-described cell cycle-dependent interactions.

The protein–DNA interactions occurring at the origin located near the lamin B2 gene, as revealed by *in vivo* footprinting, undergo an expansion/reduction cycle which is reset at every M phase. This cycle can be summarized thus (see Figure 7): in G₀, while the area corresponding to the origin is devoid of protection, the promoter of the *ppv1* gene is loaded with transcription factors, consistent with the demonstrated continuous expression of a probable housekeeping gene. As the cells move into G₁ a very

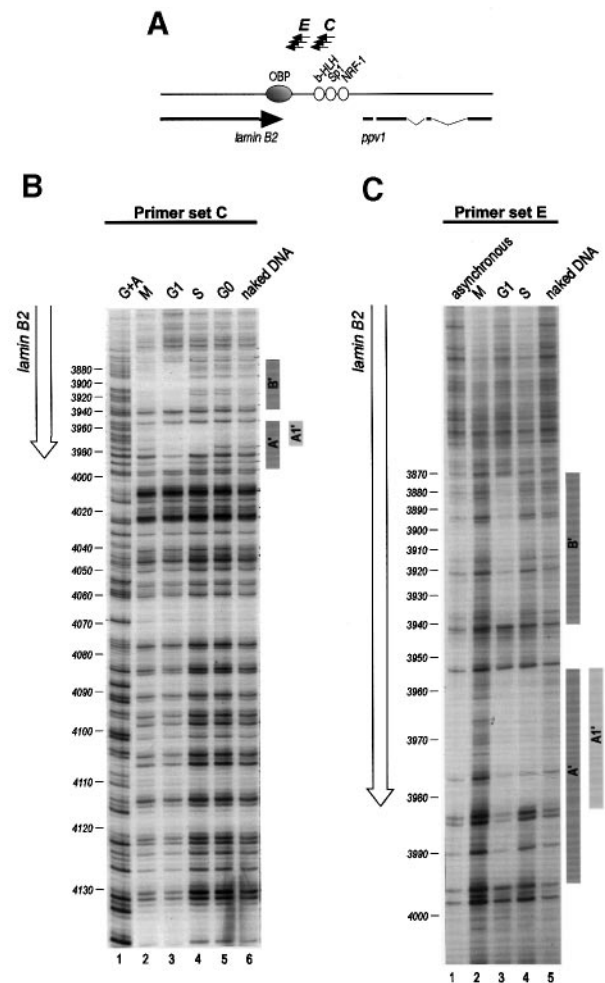


Fig. 4. DNA–protein interactions on the upper strand in the region upstream of the *ppv1* gene promoter region. (A) Primer set C was used to visualize sequences on the upper strand from nucleotides 4130 to 3860 and primer set E from nucleotides 4000 to 3850. In (B) and (C) genomic footprinting analysis was performed at different moments of the cell cycle. The light-shaded boxes indicate the region protected from methylation in S phase; the extent of the G₁-specific protections are indicated by the dark-shaded boxes.

conspicuous protection appears, which extends over 100 bp in the origin area, and causes alterations in the DMS reactivity pattern even at large distances, with the appearance of two hypersensitive sites tens of bp downstream, probably suggestive of a distortion of the chromatin structure; conversely, the promoter footprints remain unchanged. When the cells move into S, the large protection shrinks to a smaller region; again, the *ppv1* promoter footprints remain unchanged. Finally, the passage into mitosis brings the removal of all these protein–DNA interactions, both at the *ppv1* promoter and at the origin area.

The pattern of protection at the origin area is remarkably asymmetric on the two strands. The protected region is comparable in size on the two strands in G₁ cells (Figure 6), while it is significantly smaller on the upper strand in S phase.

It is of interest to note that the protection pattern which is observable in asynchronous cell populations is very similar to the pattern detectable in the S phase, both for the lower (compare the gels in Figure 1 with lane 9 in

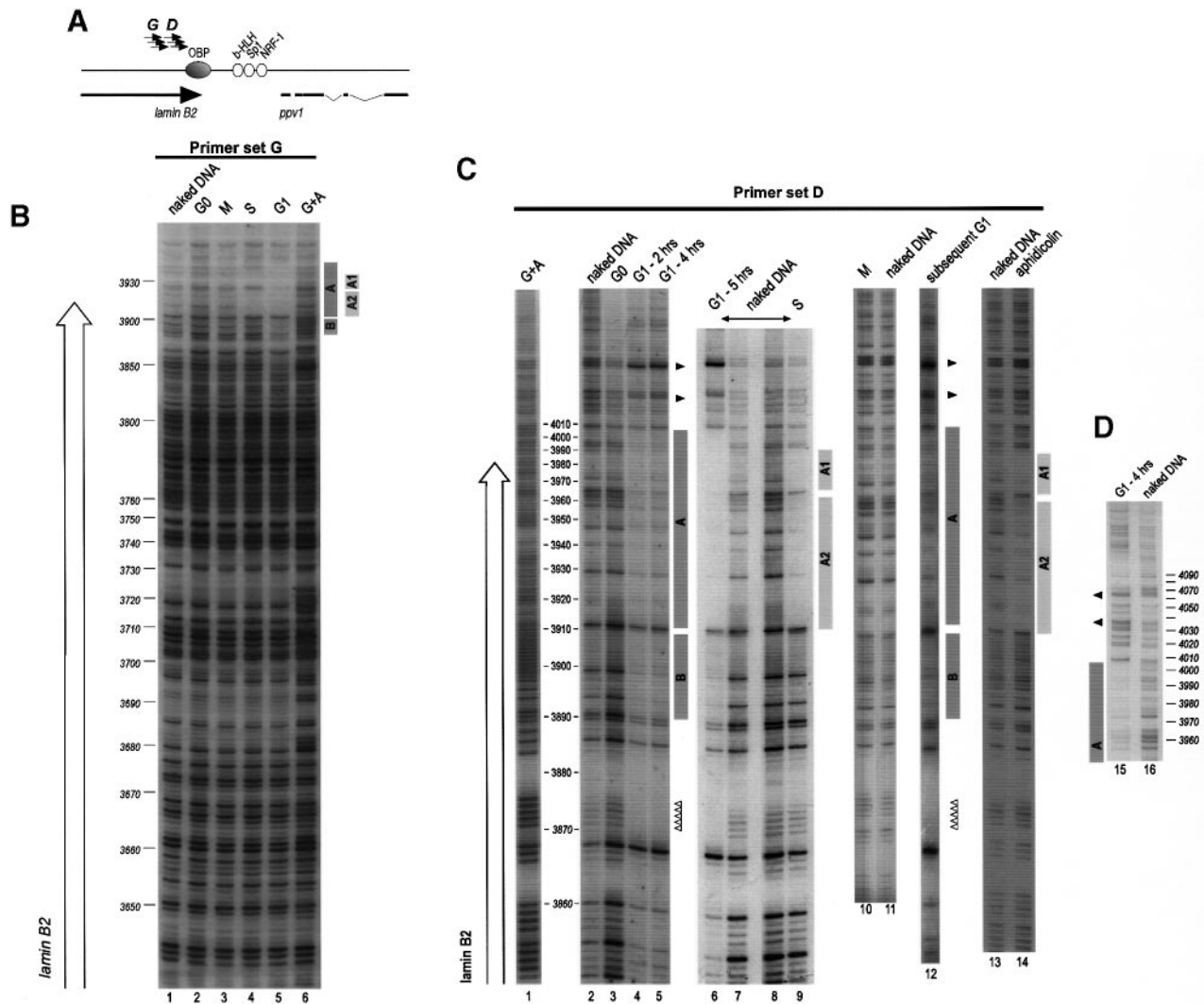


Fig. 5. DNA–protein interactions on the lower strand in the OBP region. (A) Primer set G was used to visualize sequences on the lower strand from nucleotides 3650 to 3940 and primer set D was utilized to visualize the sequences on the lower strand from nucleotides 3850 to 4090. In (B) and (C) genomic footprinting analysis was performed in different conditions, at different moments of the cell cycle; the light-shaded boxes indicate the region protected from methylation in S phase (A2 and A1). The region of more extended protection in G₁ is indicated by dark-shaded boxes (A and B); the guanine residues hyperreactive to *in vivo* DMS treatment are indicated by filled arrowheads; open arrowheads represent nucleotides hyporeactive to DMS *in vivo*. In (D) lane 15 is the same as lane 5 in (C), but the longer run of the gel allows a better resolution of the region upstream of box A.

Figure 5C) and for the upper (compare lanes 1 and 4 in Figure 4B) strands. This is not surprising if one considers that, in an asynchronous population, the fraction of cells with protection at the A1, A2 and A1' boxes will be equal to the sum of the fraction of cells in S plus the fraction of cells in late G₁ (we do not yet know whether the footprint also remains in G₂); conversely, the fraction of cells having their genome protected at boxes A, A', B and B' (not protected in S) corresponds only to the fraction of cells in late G₁. In fact, if we compare the footprints on the A and B boxes in cells that have transited increasing intervals of G₁ (Figure 5C, lanes 4–6), we observe that they are barely visible after 2 h, whereas they are quite conspicuous after 5 h. Thus, it is not surprising that in an asynchronous population the protection present in the highest fraction of genomes is prevalent. It must also be borne in mind that, since the S phase protection on the upper strand (Figure 5C, box A1') is devoid of guanosine nucleotides, this region had previously been considered

unprotected in asynchronous populations of HL-60 cells. A more thorough understanding of the reasons for the observed asymmetry between the protections of the lower and upper strands necessarily begs the identification and characterization of the protein factors interacting with this region.

The cyclic variations of the protein–DNA interactions occurring in the lamin B2 origin area during the cell cycle are reminiscent of the behaviour of the origin activation protein complex in *S.cerevisiae*. In our case, the absence of any interaction in G₀ fits with the incompetence for proliferation of these cells. In G₁, in much the same way as the large pre-RC forms on the ORC area in yeast (Diffley *et al.*, 1994), an extended protection around the OBP area appears in human cells. As the yeast cell moves into S phase and the origins are activated to fire by the pre-RC, the latter dissolves and leaves a smaller post-replication complex to mark the origin (Diffley *et al.*, 1994). In striking similarity, as the IMR-90 cell moves

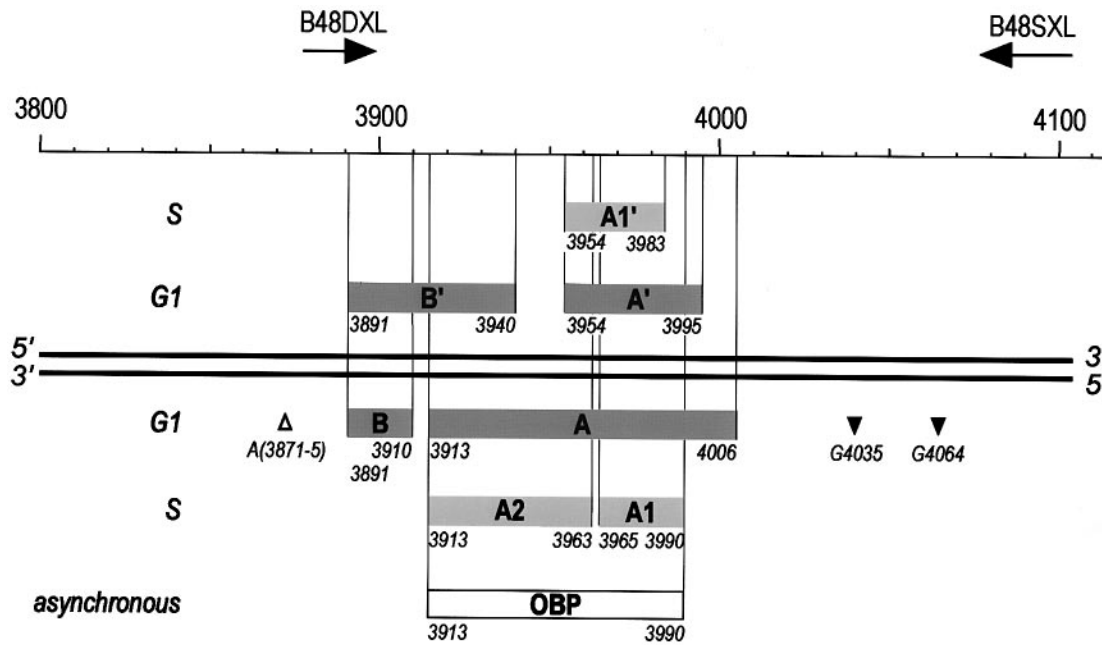


Fig. 6. Summary of the interactions in the origin zone. The positions of the nucleotides protected from methylation on the upper or lower strands are shown here for G₁ (dark-shaded boxes), for S (light-shaded boxes) and for asynchronous cells (open boxes). Nucleotides hypersensitive to DMS modification are indicated by filled arrowheads pointing down; the adenosine residues hyporeactive to methylation are indicated by an open arrowhead pointing up. The localization of the primer set B48 flanking the site of the lamin B2 origin is indicated at the top of the scheme.

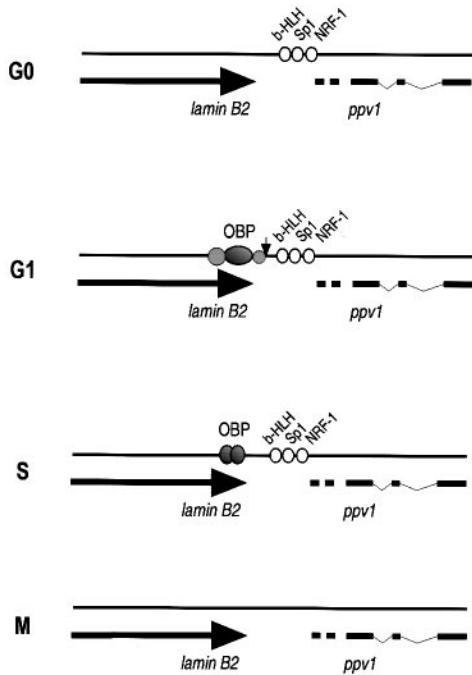


Fig. 7. Summary of the variations of the DNA-protein interactions during the cell cycle. Schematic representation of the DNA-protein interactions at the lamin B2 origin at different phases of the cell cycle. The localizations of the 3' end of the lamin B2 cDNA and of the 5' end of the *ppv1* transcript are shown.

into S phase and the lamin B2 origin (known to be one of the first origins to be activated) fires, the extended footprint present at the end of G₁ shrinks to the more compact OBP size and remains on the origin until mitosis. This transition from a large pre-replication-like complex to a smaller post-replication-like one is insensitive to the status of the growing fork, since it occurs also in conditions

in which fork progression is blocked by the presence of aphidicolin but initiation has taken place (Figure 5C, lanes 13 and 14). Furthermore, the same transition is not correlated with variations in the transcriptional level of this region, since, as already reported (Dimitrova *et al.*, 1996), terminally differentiated cells still show significant transcription of the genes immediately upstream and downstream, whereas the OBP area is not loaded in these conditions.

At first sight, the situation in mitosis is different in the yeast and human systems, since the ORC is known to remain origin-bound in yeast (Diffley *et al.*, 1994; Aparicio *et al.*, 1997; Tanaka *et al.*, 1997), whereas in human cells the OBPs leave the origin, only to bind it again in the more extended form at the next G₁. However, one must consider that, in contrast to metazoan chromosomes, yeast chromosomes are not packaged at metaphase; it is thus not surprising that in metazoans the proteins binding the origins are squeezed out when the chromosome becomes packaged to undergo the mitotic transactions. In fact, it has been shown that the ORC is at least partly displaced from chromatin during mitosis in *Xenopus* cells (Romanowski *et al.*, 1996).

With these considerations it seems reasonable to propose that the extended footprint observed in G₁ may correspond to a pre-RC, and that the more restricted one seen in S may be the human counterpart of the yeast ORC. A search for the human proteins binding *in vivo* and *in vitro* to the OBP area is now in progress.

Materials and methods

Cell culture and synchrony

The human diploid fibroblast IMR-90 (American Type Culture Collection, passage 8) and HeLa cell lines were grown in Dulbecco's modified Eagle's medium (DMEM); human lymphoblastoid Jurkat T cells and

primary human peripheral blood lymphocytes were maintained in RPMI 1640. Both media were supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 50 µg/ml gentamicin, and cultures were kept under 5% CO₂ at 37°C.

Primary human lymphocytes were isolated by Ficoll centrifugation of 50 ml of blood from a normal donor. T lymphocytes were stimulated by culture in RPMI 1640 in the presence of 1 µg/ml phytohaemagglutinin (Sigma, St Louis, MI) and 10% interleukin-2 (Cellular Products Inc., San Diego, CA).

For the synchronization experiments, IMR-90 cells were plated at 40–50% confluence and then collected in G₀ by incubation in serum-free medium for 3–4 days. Before re-entry of cells into the cell cycle, G₀ cultures were washed once with serum-free medium and then incubated in complete medium with 10% FCS for varying times (see Results).

In order to accumulate cells at the G₁/S boundaries, cultures arrested in G₀ by serum starvation were incubated in complete medium, containing 5 µg/ml aphidicolin (Sigma) for 24 h. Mid-S-phase cells were obtained by washing cells arrested at G₁/S boundary with serum-free medium followed by incubation in complete medium with 10% FCS for 5 h. After an additional 5.5 h the population of mitotic cells was enriched by collection of non-adherent cells obtained by gentle shaking of the cell culture flasks. The same cell population was also used to obtain cells entered in the subsequent G₁ phase after plating in complete medium for 8 h.

A schematic representation of these procedures is shown in Figure 2.

Flow-cytometric analysis

Approximately 10⁶ cells were harvested, fixed with 70% ethanol for at least 1 h at 4°C, washed with 1 ml of ice-cold phosphate-buffered saline (PBS), digested for 20 min by adding 100 µl of 0.5 µg/ml RNase A (Boehringer Mannheim, Germany) and 100 µl of 1% Nonidet P-40 (NP-40), stained with 1 ml of propidium iodide (50 µg/ml in PBS) and scanned on a Becton Dickinson FACScan analyser; the results were plotted using Cellfit software.

Genomic footprinting

For each genomic footprinting experiment, 10⁷ cells were treated with 0.1% DMS (Aldrich) for 5 min at room temperature. The reaction was stopped by the addition of 5 ml ice-cold DMS stop solution (1% bovine serum albumin and 100 mM β-mercaptoethanol dissolved in PBS). The cells were collected, washed once more with DMS stop solution and twice more with PBS. Finally the cells were re-suspended in 1 ml of RSB (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl₂) and incubated on ice for 10 min. An equal volume of 0.2% NP-40 in RSB was added followed by incubation for 10 min at 4°C.

DNA was isolated from pelleted nuclei and piperidine cleavage at methylated bases was performed as described previously (Mueller et al., 1992). Sequenced and footprinted DNA was analysed by ligation-mediated PCR according to the protocol of Quivy and Becker (1993). Sequencing reactions for genomic DNA were performed as described (Saluz and Jost, 1987). The three sets of oligonucleotide primers used for the upper strand (sets A, C and E) and the two sets for the lower strand (sets D and G) have been described previously (Dimitrova et al., 1996).

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