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In vivo protein–DNA interactions at a human DNA replication origin

(lamin B2/origin-binding proteins/ligation-mediated PCR/genomic footprinting)

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ABSTRACT **Protein–DNA interactions were studied *in vivo* at the region containing a human DNA replication origin, located at the 3′ end of the lamin B2 gene and partially overlapping the promoter of another gene, located downstream. DNase I treatment of nuclei isolated from both exponentially growing and nonproliferating HL-60 cells showed that this region has an altered, highly accessible, chromatin structure. High-resolution analysis of protein–DNA interactions in a 600-bp area encompassing the origin was carried out by the *in vivo* footprinting technique based on the ligation-mediated polymerase chain reaction. In growing HL-60 cells, footprints at sequences homologous to binding sites for known transcription factors (members of the basic-helix–loop–helix family, nuclear respiratory factor 1, transcription factor Sp1, and upstream binding factor) were detected in the region corresponding to the promoter of the downstream gene. Upon conversion of cells to a nonproliferative state, a reduction in the intensity of these footprints was observed that paralleled the diminished transcriptional activity of the genomic area. In addition to these protections, in close correspondence to the replication initiation site, a prominent footprint was detected that extended over 70 nucleotides on one strand only. This footprint was absent from nonproliferating HL-60 cells, indicating that this specific protein–DNA interaction might be involved in the process of origin activation.**

Genomes of higher eukaryotes consist of approximately 10^4 to 10^5 tandemly arranged replicons (1–3) which are activated in a spatially and temporally regulated fashion (reviewed in ref. 4). The first eukaryotic replication origins (*ori*s) were identified in the yeast *Saccharomyces cerevisiae* by the application of the assay for autonomously replicating sequences (ARSs). *In vitro* studies of the protein–DNA interactions occurring at the yeast *ori*s allowed the identification and purification of a multiprotein complex (origin recognition complex, ORC) binding to ARS1 in an ATP-dependent manner and generating footprints very similar to those obtained *in vivo* (5–7). The binding of ORC to ARSs correlates with their functional activity, making ORC a strong candidate for a eukaryotic initiator protein complex (8–10).

The study of *ori*s in higher eukaryotes has proven much more arduous and is still open to controversy (reviewed in ref. 11). Over the past years, we have been able to identify an *ori* located in an actively transcribed area in the subtelomeric G-negative band p13.3 of human chromosome 19 (12, 13). Thanks to the development of a highly sensitive *ori*-mapping technique, the precise DNA replication start site was located within an ≈500-bp region encompassing the 3′ noncoding end of the lamin B2 gene (*LMNB2*) and the nontranscribed spacer preceding another still uncharacterized gene, provisionally named *ppv1* (13–15). The same origin, originally identified in HL-60 cells, was found to be active also in several other human cell types, including activated peripheral lymphocytes (S. Kumar, M.G., G.B., S.R., and A.F., unpublished results). A map of the 13.7-kb genomic fragment containing the replication origin is shown in Fig. 1*A*.

The finding that the lamin B2 *ori* is located in a highly transcribed region is not surprising, since numerous reports have implicated transcriptional regulatory elements in the control of DNA replication (16–19). Moreover, the domain is replicated at the very beginning of the S phase (12), consistent with the well-documented correlation between transcriptionally active genomic regions and early replication.

We have now addressed the study of the specific protein–DNA interactions at the lamin B2 *ori*. To this purpose, we have exploited the *in vivo* footprinting technique based on the ligation-mediated polymerase chain reaction (LMPCR) (20). In the past few years we have extensively utilized this technique (21), which, in comparison with *in vitro* binding studies, is particularly suitable for the detection of the protein–DNA interactions actually occurring in the nucleus, since it is sensitive to the accessibility of sites to protein factors and possible epigenetic modification of DNA such as methylation. Furthermore, we have taken advantage of the property of HL-60 myeloid cells to undergo terminal differentiation upon chemical stimulation (22), a process which is accompanied by a complete arrest of DNA replication (16). Thus, any specific aspect of the chromatin structure in proliferating cells can be compared with the replicationally "null" state of the differentiated cells. The results reported here indeed show evidence for specific replication-correlated interactions at the lamin B2 *ori*.

MATERIALS AND METHODS

Cell Cultures. HL-60 (human promyelocytic leukemia) cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and gentamycin at 50 μg/ml in a 5% CO_2 atmosphere at 37°C. When appropriate, differentiation was induced by the addition of retinoic acid (Sigma) at a final concentration of 10^{-6} M to a culture of 5×10^5 cells per ml, followed after 24 hr by the addition of dimethylformamide (Merck) at a final concentration of 60 mM. Viability was assessed by the ability of the cells to exclude trypan blue dye. Differentiated cells were used after a 72-hr

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Abbreviations: *ori*, DNA replication origin; ORC, origin recognition complex; LMPCR, ligation-mediated polymerase chain reaction; DMS, dimethyl sulfate; DHS, DNase I-hypersensitive site(s); OBP, origin-binding protein(s); b-HLH, basic-helix–loop–helix; NRF-1, nuclear respiratory factor 1; UBF, upstream binding factor.

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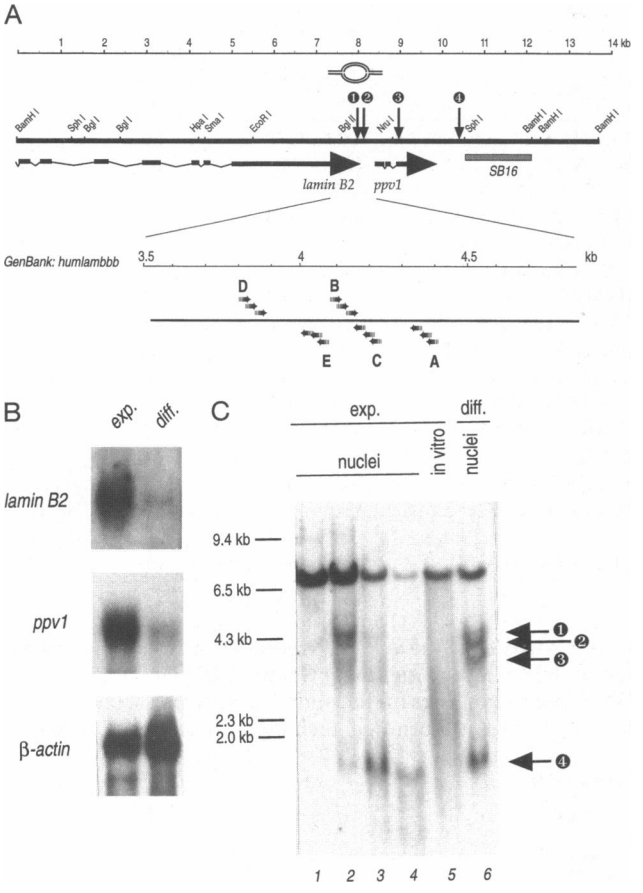

FIG. 1. Transcription pattern and nuclease sensitivity of the lamin B2 genomic region in human HL-60 cells. (*A*) Schematic representation of the lamin B2 gene domain. The bubble structure marks the position of the replication origin. The two transcripts are depicted and the positions of the DNase I-hypersensitive sites (DHS) are indicated by vertical arrows. In the bottom part, the *ori*-encompassing region is expanded and the locations of the oligonucleotide primer sets for the LMPCR are shown. (*B*) Northern blot analysis of the transcription pattern in the lamin B2 chromosomal region. Blots of mRNA isolated from exponentially growing and differentiated HL-60 cells were hybridized to probes specific for the lamin B2, *ppv1*, and β-actin genes. (*C*) Mapping of DHS in the lamin B2 domain. Nuclei from exponentially growing (lanes 1–4) or differentiated (lane 6) HL-60 cells were treated with increasing concentrations of DNase I (0–10 units/ml) and DNA was then purified. Naked DNA was digested *in vitro* with DNase I and served as a control (lane 5). The different DNA preparations were then digested with *Bam*HI and *Eco*RI, electrophoresed on 1% agarose gels, transferred to a nitrocellulose membrane, and hybridized to the 1.7-kb SB16 probe. The subfragments resulting from the DNase I digestion are indicated by arrows. The positions of the molecular weight marker fragments (λ DNA digested with *Hin*dIII) are indicated on the left side of the gel.

incubation in retinoic acid/dimethylformamide-containing medium.

Preparation of Nuclei. HL-60 cells were washed once with cold phosphate-buffered saline (PBS), resuspended in RSB (10 mM Tris·HCl, pH 7.4/10 mM NaCl/3 mM $MgCl_2$) at 2.5 × 10^7 cells per ml, and incubated on ice for 5 min. An equal volume of 0.2% Nonidet P-40 in RSB was added and the cell suspension was incubated in ice for 3–5 min after gentle mixing. Nuclei were pelleted, washed once with RSB, and resuspended in RSB at 2.5 × 10^7 nuclei per ml. Aliquots of the isolated nuclei were adjusted to DNase I buffer (25 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$/1 mM $CaCl_2$, final concentrations) and DNase I digestion [DNase I (Boehringer Mannheim) at 0.1–5 units/ml] was allowed to proceed for 10 min in ice, after which nuclei were lysed and DNA was extracted as described in ref. 23.

LMPCR. The procedures for *in vivo* and *in vitro* methylation by dimethyl sulfate (DMS), piperidine cleavage of methylated DNA, DNA sequencing reactions, and the LMPCR were performed as described (20, 24, 25). The nucleotide sequences and the amplification conditions for each primer are described in Table 1.

Southern Blotting and Hybridization. HL-60 nuclei were digested with DNase I at increasing concentrations (0.5–10 units/ml), and DNA was purified and was digested to completion with the appropriate restriction enzymes, extracted with phenol/chloroform, and precipitated with ethanol. Samples (≈30 μg) of DNA from individual DNase I reactions were analyzed on 0.8–1% agarose gels in Tris–acetate buffer, pH 8, at 1.5 V/cm, overnight. DNA was transferred to nitrocellulose (Hybond C; Amersham) or nylon membranes (Nytran; Schleicher & Schuell) and hybridized with the appropriate probes as described (23).

Northern Blot Analysis. Total RNA was extracted from HL-60 cells as described (26). mRNA was prepared by using an Oligotex-dT mRNA kit (Qiagen, Chatsworth, CA). Blotting and hybridization were performed as described (23).

RESULTS AND DISCUSSION

Transcriptional Features of the Lamin B2 Chromosomal Domain. In a previous report, we demonstrated that the differentiation of HL-60 cells by treatment with retinoic acid and dimethylformamide results in the inactivation of the lamin B2 *ori* (16). We subsequently investigated the effect of the same treatment on the transcription pattern in the lamin B2 locus. We found that differentiation of HL-60 cells is also accompanied by a strong reduction of the levels of the lamin B2 and *ppv1* transcripts (Fig. 1*B*). We will show below that the relative intensity of the protein–DNA interactions observed just upstream of the *ppv1* gene is diminished in a parallel fashion in differentiated cells.

Chromatin Structure of the Human Lamin B2 Chromosomal Domain. To determine whether the lamin B2 *ori* lies in a region characterized by an altered chromatin structure, we examined its sensitivity to DNase I cleavage. To this end, nuclei isolated from exponentially growing or differentiated HL-60 cells were treated with increasing concentrations of DNase I. DNA was purified and digested to completion with *Bam*HI and *Eco*RI, and the double-stranded DNase I-cleavage sites were mapped by indirect end-labeling (27, 28) from the right end of the *Bam*HI/*Eco*RI restriction fragment (probe SB16) (see Fig. 1*A*). Four DHS were revealed in the lamin B2 chromosomal region in nuclei from exponentially growing HL-60 cells (Fig. 1*C*, lanes 1–4). DNase I treatment of naked HL-60 DNA produced a uniform smear of digestion products (Fig. 1*C*, lane 5). The positions of the four DHS were confirmed by using different combinations of restriction enzymes and hybridization probes (data not shown). Interestingly, all the hypersensitive sites are constitutive, since they are also present in the chromatin of terminally differentiated HL-60 cells (Fig. 1*C*, lane 6).

Two of the nuclease-hypersensitive sites (DHS-1 and DHS-2) map to the spacer between the lamin B2 and the *ppv1* genes, exactly where the replication initiation site is located. The data indicate an altered, highly accessible chromatin structure therein, typical of the DNA domains involved in the control of transcription, replication, or recombination (29, 30). In particular, the assembly of DNA into nucleosomes is inhibitory for the initiation of DNA replication (31–34), and overcoming this inhibitory effect may require specific protein–DNA interactions at the replication origins. Replication origins in yeast (35, 36) and *Tetrahymena* (37) are maintained in an "open" chromatin conformation in growing and resting

Table 1. Sequences and amplification conditions for primers

Set	Primer	Length, bp	Sequence	Genomic position	Annealing temperature, °C
A	1	25	5′-TTACCTACACGAGCTACCCGTGGTT-3′	4385–4409	60
	2	22	5′-TGGTTGCGACTCCGCGGGAAGA-3′	4368–4389	68
	3	27	5′-TCCGCGGGAAGAGGGAGGCCCTGACTT-3′	4353–4379	70
B	1	28	5′-GGCTAGTGTAGCTAGTGTAAACAGGACC-3′	4091–4118	60
	2	25	5′-GTAAACAGGACCCAGGCGATGCATG-3′	4107–4131	67
	3	27	5′-CAGGACCCAGGCGATGCATGGGACCCT-3′	4112–4138	70
C	1	25	5′-TCGCATCACGTGACGAAGAGTCAGC-3′	4179–4203	60
	2	25	5′-GAGTCAGCTTGTGCAACAGCGTCGG-3′	4162–4186	66
	3	27	5′-GCTTGTGCAACAGCGTCGGAGGCTCAC-3′	4154–4180	68
D	1	25	5′-GTCACAGCACAACCTGCAAAAACGG-3′	3795–3821	60
	2	25	5′-CAAAAACGGAGCTGGGCTGCAGCTG-3′	3813–3837	70
	3	26	5′-GGGCTGCAGCTGGGGCTGGCATGGAC-3′	3826–3851	72
E	1	25	5′-GGGGTGGAGGGATCTTTCTTAGACA-3′	4049–4074	60
	2	27	5′-GACATCCGCTTCATTAGGGCAGAGGCC-3′	4026–4052	68
	3	27	5′-TCATTAGGGCAGAGGCCCGGCTCGAGC-3′	4016–4042	70
SL*		11	5′-GAATTCAGATC-3′		
LL*		25	5′-GCGGTGACCCGGGAGATCTGAATTC-3′		68

*SL and LL are the short and long linker primers, respectively.

cells. Our observation that the DHS are maintained in differentiated HL-60 cells is consistent with the idea that a replication-favorable chromatin structure is preserved even in nonreplicating cells.

Fine Analysis of the *in Vivo* Protein–DNA Interactions at the Lamin B2 *ori* in Exponentially Growing HL-60 Cells. Protein–DNA interaction sites in the lamin B2 *ori* region were analyzed by the *in vivo* footprinting technique based on LMPCR as

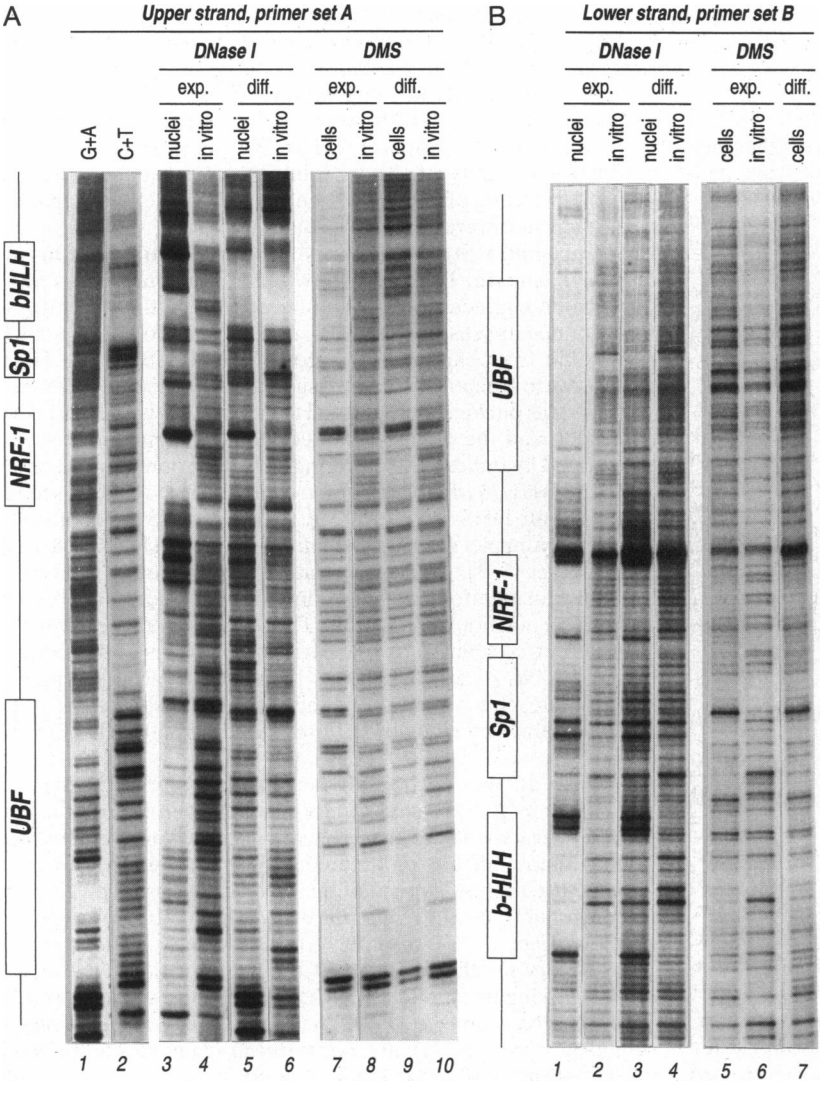

FIG. 2. Genomic footprinting at the human lamin B2 replication origin. (*A*) Primer set A was used to visualize fragment ladders on the upper DNA strand in exponentially growing (lanes 3, 4, 7, and 8) and differentiated (lanes 5, 6, 9, and 10) HL-60 cells. Lanes 3–6, DNase I digests; lanes 7–10, DMS modifications. Control reactions with naked DNA are shown in lanes 4, 6, 8, and 10. The G+A and C+T sequencing reaction products are shown in lanes 1 and 2, respectively. (*B*) Primer set B was used to visualize fragment ladders on the lower DNA strand in exponentially growing (lanes 1, 2, 5, and 6) and differentiated (lanes 3, 4, and 7) HL-60 cells. Lanes 1–4, DNase I digests; lanes 5–7, DMS modifications. Control reactions with naked DNA are shown in lanes 2, 4, and 6.

developed by Mueller and Wold (20, 38). Two DNA-nicking reagents were used, DNase I (which can be utilized only with isolated nuclei) and DMS (a small molecule that can be used with intact cells). The combined use of these two treatments, by providing complementary information, is best suited for obtaining a fine description of protein–DNA interactions.

Exponentially growing (or differentiated—see next paragraph) HL-60 cells were treated with DMS at increasing concentrations, and DNA was purified and then cleaved at the modified bases by treatment with piperidine. Alternatively, nuclei were first prepared and subsequently digested with DNase I at increasing concentrations, after which DNA was isolated. Naked DNA, prepared from untreated HL-60 cells, was digested with DNase I or methylated *in vitro* to an extent similar to that *in vivo* and was used as a control. The various DNA species were then subjected to LMPCR and the amplification products were visualized by autoradiography after separation on 6% sequencing gels. Five sets, each of three sequence-specific primers, were used, spanning an ≈600-bp region in the 3′ end of the lamin B2 gene and the adjacent spacer. The location and orientation of the primers are shown in Fig. 1*A*, and the nucleotide sequence and conditions for amplification are described in Table 1.

We have previously shown (12, 39) that the intergenic promoter just downstream of the lamin B2 *ori* contains a sequence recognized and bound by a protein belonging to the B subgroup of the basic-helix–loop–helix (b-HLH) family (including the Myc protein and the transcription factor USF). We examined, accordingly, the region between nucleotides 4150 and 4400 (see the sequence displayed in Fig. 4) by LMPCR footprinting. Primer sets A and B were used to map single-strand cuts on the upper and lower strand, respectively, and the results obtained are displayed in Fig. 2. Multiple protected areas are visible in this region in exponentially growing HL-60 cells. The b-HLH motif is embedded in a 20-bp sequence (nucleotides 4186–4205) which is protected on both strands from the action of DNase I and DMS. The protected site is flanked on both sides by hypersensitive nucleotides.

Another footprint is adjacent to the b-HLH site, covering the region between nucleotides 4216–4231 on the upper strand and 4211–4230 on the lower strand. The left side of the footprint (nucleotides 4211–4221) contains a high-affinity binding site, 5′-GCTCCGCCCCGG-3′, for transcription factor Sp1 (40).

Further downstream, a prominent footprint is evident on both strands in the DNase I- and DMS-cleavage patterns. It contains a sequence, 5′-TGCGCATGCGCG-3′, which matches perfectly the binding site of the transcription factor nuclear respiratory factor 1 (NRF-1) (41). Preliminary experiments (P. Norio, M.G., G.B., S.R., and A.F., unpublished data) suggest that NRF-1 is indeed binding to this region.

Finally, protein binding *in vivo* was detected ≈30 bp downstream from the NRF-1 motif, over nucleotides 4304–4352. This site contains three short, directly repeated sequences, 5′-(T/C)CGGCC-3′, which are homologous to a binding site for the ubiquitous RNA polymerase I transcription factor upstream binding factor (UBF) (42).

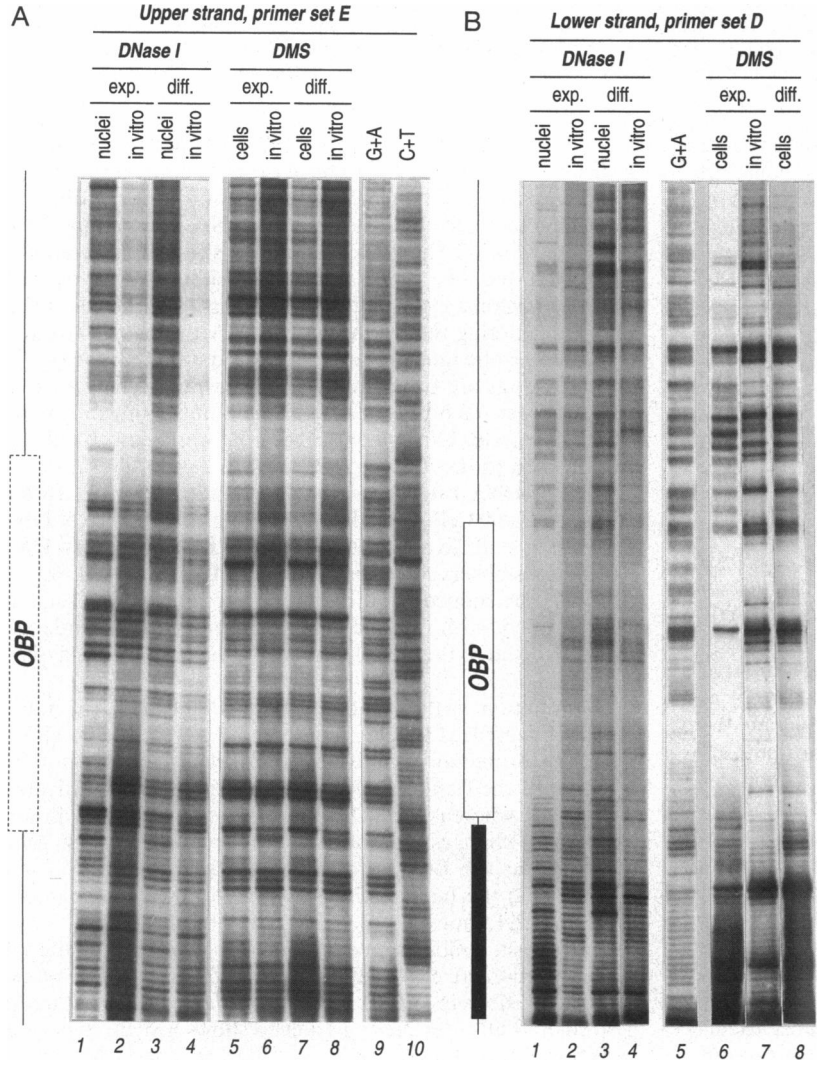

FIG. 3. Genomic footprinting at the human lamin B2 replication origin. (*A*) Primer set E was used to visualize fragment ladders on the upper DNA strand in exponentially growing (lanes 1, 2, 5, and 6) and differentiated (lanes 3, 4, 7, and 8) HL-60 cells. Lanes 1–4, DNase I digests; lanes 5–8, DMS modifications. Products of control reactions with naked DNA are shown in lanes 2, 4, 6, and 8. The G+A and C+T sequencing reaction products are shown in lanes 9 and 10, respectively. The position corresponding to the origin-binding protein (OBP) site protected on the other strand is indicated in the dotted box on the left. (*B*) Primer set D was used to visualize fragment ladders on the lower DNA strand in exponentially growing (lanes 1, 2, 6, and 7) and differentiated (lanes 3, 4, and 8) HL-60 cells. Lanes 1–4, DNase I digests; lanes 6–8, DMS modifications. Products of control reactions with naked DNA are shown in lanes 2, 4, and 7. The G+A sequencing reaction products are shown in lane 5. The position corresponding to the OBP site is indicated in the box on the left; the adjacent black box shows the position of the hypersensitive A+T-rich region.

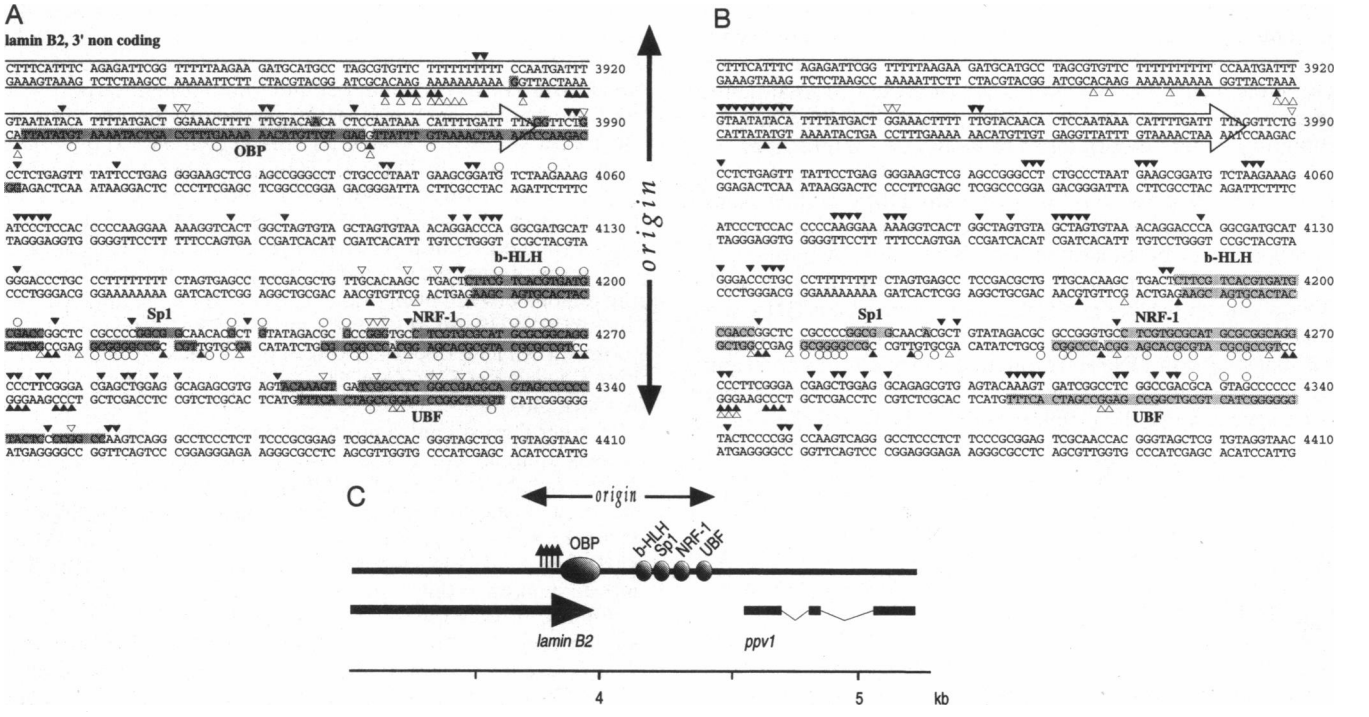

FIG. 4. Summary of the *in vivo* footprinting data for exponentially growing (*A*) and differentiated (*B*) HL-60 cells. Sequences protected from DNase I cleavage are indicated by shaded boxes and DNase I-hypersensitive nucleotides, by filled arrowheads. Nucleotides protected from methylation are marked by open circles and nucleotides hypersensitive to DMS modification, by open arrowheads. The end of the lamin B2 cDNA is indicated by the long open horizontal arrow. (*C*) Schematic representation of the protein–DNA interactions at the lamin B2 *ori*. The location of the replication origin is indicated and the 3′ end of the lamin B2 cDNA and the 5′ end of the *ppv1* transcript are shown. The cluster of arrows pointing upwards indicates the position of the A+T-rich region which becomes DNase I-hypersensitive upon OBP binding. Numbering refers to file humlambbb of GenBank.

It appears likely that all these protein–DNA interactions are involved in the promoter activation of the *ppv1* gene. In addition, these interactions might also have a role in chromatin structure and in replication origin activation, the more so since the b-HLH motif could conceivably be recognized by the Myc–Max complex, which is believed to be involved in DNA replication regulation. Incidentally, Myc is also the main amplified oncogene of the HL-60 cells used in this study.

Examination of the region immediately upstream of the b-HLH motif (upper strand), using primer set C, did not reveal any footprint in this area (data not shown).

Extension of the LMPCR analysis, using primer set D, revealed a large and clear footprint on the lower strand, over nucleotides 3923–3992 (Fig. 3*B*, lanes 1 and 6). The footprint extends over 70 nucleotides and has a bipartite structure with two highly protected sites separated by a group of less protected nucleotides containing a hypersensitive nucleotide in the middle. It should be noted that this footprint maps exactly to the region previously shown to contain the site for initiation of bidirectional DNA replication (16). Considering the large size and bipartite structure of the protected sequence, it is likely that more than one protein binds there. We designate the nuclear activity responsible for the footprinting of the lamin B2 replication origin OBP [origin-binding protein(s)]. It is noteworthy that the binding of the protein(s) to DNA induces a structural deformation in the adjacent A+T-rich region which makes it hypersensitive to DNase I cleavage (seen in the lower portion of the autoradiograph displayed in Fig. 3*B*, lane 1). Interestingly, the LMPCR *in vivo* footprinting analysis of the upper strand (primer set E) failed to demonstrate protection at the same site, except for a few protected single nucleotides (Fig. 3*A*, lanes 1 and 5), indicating that the protein–DNA contact, even though very extended, involves almost exclusively the lower strand. A computer search of known protein recognition sequences (October 1995) did not reveal any significant homology to the 70-bp footprint discovered here.

A comparison of the extent of protection over the different footprints indicates that the protein–DNA interactions differ in strength. While the NRF-1 and OBP sites display complete protection, the Sp1, b-HLH, and UBF sites are only partially protected against either DNase I cleavage or DMS modification. Considering that we have worked with nonsynchronized cell cultures, one interpretation of these data is that the NRF-1 and OBP sites are occupied during the whole cell cycle in all cells, whereas the other sites could be bound during only a part of the cell cycle. Experiments with synchronized cell cultures can be used to test this hypothesis.

Protein–DNA Interactions at the Lamin B2 *ori* in Differentiated HL-60 Cells. To address whether the absence of DNA replication leads to alterations in the pattern of protein–DNA interactions observed in proliferating HL-60 cells, HL-60 cell cultures were induced to terminal differentiation by a sequential treatment with retinoic acid and dimethylformamide and then subjected to the DMS- and DNase I-sensitivity analysis as described above.

Examination of the b-HLH, Sp1, NRF-1, and UBF motifs reveals a decreased level of protection in differentiated HL-60 cells; the protection against DNase I cleavage (but not the intensity of the flanking hypersensitive sites) was significantly decreased, whereas the DMS-modification pattern showed only minor changes. Although these variations suggest some alteration in the DNA–protein contacts in the region, it is obvious that the factors still bind *in vivo* to their recognition sites (Fig. 2*A*, lanes 5 and 9; Fig. 2*B*, lanes 3 and 7).

In contrast, a most impressive effect is produced by the cell differentiation at the OBP site (Fig. 3*B*). The prominent footprint visible in proliferating cells has completely disappeared, and DNA in the region even shows a slight hypersensitivity to DNase I digestion *in vivo* (Fig. 3*B*, lane 3). Moreover,

in the absence of protein binding, the normal DNase I sensitivity of the A+T-rich region adjacent to the OBP site is restored.

The data from the LMPCR *in vivo* footprinting analysis in exponentially growing and differentiated HL-60 cells are summarized in Fig. 4.

Concluding Remarks. The large and prominent footprint found in the *ori* region, nearest to the replication initiation site which was previously identified, is of particular interest for the objective of the present study. The features of this footprint can be summarized as follows: (*i*) The protection covers 70 nucleotides on the lower DNA strand only; no protection, apart from a few protected single nucleotides, was observed on the upper DNA strand. (*ii*) The footprint is divided in two parts by an "island" of less-protected nucleotides, containing a hyperreactive nucleotide in its center. (*iii*) Periodic hypersensitive nucleotides, spaced at 11- to 12-bp intervals, were detected in the DNase I-cleavage pattern on the upper strand, a possible indication that the secondary structure of DNA in the region deviates from the B type. (*iv*) The binding of the protein to this site *in vivo* distorts the adjacent A+T-rich region. (*v*) The protection is complete, which implies that the protein remains stably bound during most of the cell cycle in all cells. (*vi*) No sequence homology to other protein factor binding sites was found for this footprint. (*vii*) From a functional viewpoint, the most relevant feature is that the protection of the entire region disappears when HL-60 cells cease replication.

Many of the listed features are consistent with the properties of known (or candidate) proteins. The large size of the protected area suggests that more than one protein may generate the footprint. The induction of structural deformations of adjacent A+T-rich regions of DNA is considered a hallmark of the interactions of the initiation proteins with DNA at the origins.

The complete protection observed *in vivo* over the entire 70-bp region indicates that the protein(s) are bound throughout the whole cell cycle, as was previously reported for the yeast ORC (6, 43). However, if small changes in the observed interactions occur only during a short fraction of the cell cycle, they would escape our detection. Genomic footprinting with HL-60 cells, synchronized at different stages of the cell cycle, should provide an answer to this question.

Considering our observation, as well as observations reported for other eukaryotes, it is tempting to speculate that the eukaryotic replication origins might be regulated at several levels. First, an "open" chromatin structure, permissive for the initiation of DNA replication, is maintained in the vicinity of the replication origins. Second, *ori*s are marked throughout the cell cycle by binding to specific protein factors. Third, the activation of the origins proceeds through additional interactions with cell-cycle-specific factors, culminating in the formation of stable initiation complexes and the onset of DNA synthesis. It is likely that nuclear structural components, which remain obscure at present, play an important role at all these regulatory levels. Additionally, the OBP are likely to be targets of the cell cycle regulatory apparatus and could represent the final element of the cascade initiated by the interaction of growth factors with the cell membrane.

Understanding the details of these specific nucleic acid–protein interactions will require the isolation of the proteins involved and the development of suitable *in vitro* systems.

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