Gene positional changes relative to the nuclear substructure correlate with the proliferating status of hepatocytes during liver regeneration

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ABSTRACT

In the interphase nucleus the DNA of higher eukaryotes is organised in loops anchored to a proteinaceous substructure variously named but commonly known as the nuclear matrix. Important processes of nuclear physiology, such as replication, transcription and processing of primary transcripts, occur at macromolecular complexes located at discrete sites upon the nuclear substructure. The topological relationships between gene sequences located in the DNA loops and the nuclear substructure appear to be non-random, thus posing the question of whether such relationships remain invariant or change after the critical nuclear transitions associated with cell proliferation and tissue regeneration *in vivo*. The hepatocytes are cells that preserve a proliferating capacity that is readily displayed after partial ablation of the liver, leading to liver regeneration in experimental animals such as the rat. Using this animal model coupled to a recently developed PCR-based method for mapping the position of specific DNA sequences relative to the nuclear substructure, we provide evidence that transient changes in the topological relationships between specific genes and the nuclear substructure occur during liver regeneration and that such changes correlate with the actual proliferating status of the cells, thus suggesting that specific transitions in the higher-order DNA structure are characteristic of the quiescent (G0) and replicating (S) phases of the cell cycle *in vivo*.

INTRODUCTION

In the interphase nucleus the DNA of higher eukaryotes is organised in loops topologically constrained and supercoiled anchored to a proteinaceous substructure that has been

variously named depending on the isolation method as: nuclear scaffold, nuclear matrix, nuclear cage, nuclear skeleton. However, the term nuclear matrix (NM) is now widely used and loosely subsumes all others (1–3). The ultimate composition of the nuclear substructure is still a matter of debate; however, there is ample evidence suggesting that there is indeed a NM (3–5). The DNA loops are attached to the substructure by means of non-coding sequences known as matrix attachment regions (MARs), but not all potential MARs are actually involved in the DNA loop anchorage regions or LARs (6). There is evidence suggesting that MARs attached to the NM constitute boundaries of independently controlled chromatin units within which the DNA packaging and function may be changed without affecting the neighbouring regions (6–10). Several reports suggest that actively transcribed genes and early-replicating DNA sequences are closely associated with the NM or located proximal to an anchoring point to the matrix, while non-expressed genes and late-replicating regions are relatively far away from the NM (11–17). Moreover, several important processes of nuclear physiology, such as replication, transcription and processing of primary transcripts seem to occur at macromolecular complexes located at discrete sites upon the NM (18–22). Thus, the topological relationship between gene sequences located in the DNA loops and the NM appears to be very important for appropriate nuclear physiology. For example, productive infection by herpes simplex virus type 1 induces a wholesale alteration of the higher-order structure of the host-cell chromatin, resulting in the loss of DNA-loop supercoiling and organisation that correlates with the complete inhibition of host-cell DNA replication and transcription (23–25). Indeed, correct repair of DNA damage must include recovery of both the double helix integrity and the complex DNA topology, otherwise the cell will not survive (24,26). Moreover, there is important evidence suggesting that the position of whole chromosomes within the interphase nucleus is not random and that such positioning may be important for gene regulation and function (27–31). Thus, the apparent non-randomness of the topological relationships between DNA and the nuclear substructure poses the question of whether such

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relationships remain invariant or change after the critical nuclear transitions associated with cell proliferation and tissue regeneration *in vivo*. The hepatocytes are cells that rarely divide, yet they preserve a proliferating capacity that is readily displayed *in vivo* after partial ablation of the liver, that leads to liver regeneration with full recovery of liver mass and functions with subsequent return to quiescence of the hepatocytes (32). Indeed, partial hepatectomy resulting in the removal of ~70% of the liver is widely used for studies of liver growth in experimental animals such as the rat (33). We have used this animal model coupled to a recently developed PCR-based method for directly mapping the position of specific DNA sequences relative to the nuclear substructure in NM-bound templates (34), in order to explore the question of whether several genes that show differential levels of activity in the liver and belong to different chromosomes, may change or modify their relative position to the nuclear substructure during different stages of liver regeneration. Hereunder, we provide evidence that transient changes in the relative position of specific genes to the nuclear substructure occur during the process of liver regeneration, but also that most such changes correlate with the replicating status of the cells.

MATERIALS AND METHODS

Partial hepatectomy

All procedures involving animals were carried out in accordance with the Institutional Guide for Ethical Animal Experimentation (National Autonomous University of Mexico). Male Wistar rats weighing 200–250 g were used. The animals were fed water and food *ad libitum*. Surgical removal of two-thirds of the liver (mechanical partial hepatectomy) was performed between 7 and 9 am, under ether anaesthesia (35). Rats were sacrificed using ether anaesthesia at 0, 2, 24, 48, 96 h and 7 days after mechanical hepatectomy. In the control non-hepatectomised group, $n = 6$ rats. In each of the post-hepatectomy (PH) groups from 2 h to 7 days, $n = 3$ rats.

Hepatocytes

Before the hepatocyte isolation the livers were washed *in situ* by perfusion with PBS without Ca^{2+} and Mg^{2+} at 37°C for 5 min at 15 ml/min using a cannula (an 18-calibre needle) introduced in the portal vein or a main portal branch. The vena cava was cut at the level of the kidneys in order to elute the liver content. Next the tissue was perfused with a solution of collagenase IV, Sigma (0.025% collagenase with 0.075% of CaCl_2 in HEPES buffer, pH 7.6) for 15 min. The rest of the hepatocyte isolation was carried out as described (36). Viable hepatocytes were counted in a haemocytometer and used immediately for preparing nucleoids.

Preparation of nucleoids containing a nuclear substructure

The DNA loops plus the nuclear substructure constitute a 'nucleoid', a very large nucleoprotein aggregate generated by gentle lysis of a cell at pH 8 in non-ionic detergent and the presence of high salt concentration. Nucleoids were prepared as previously described (1,34). Freshly isolated and washed hepatocytes were suspended in ice-cold PBS without Ca^{2+} and

Mg^{2+} (PBS-A). Aliquots of 50 μl containing 3.5×10^5 cells were gently mixed with 150 μl of a lysis solution containing 2.6 M NaCl, 1.3 mM EDTA, 2.6 mM Tris, 0.6% Triton X-100, pH 8.0. After 20 min at 4°C, the mixture was overlaid on sucrose step gradients containing 0.2 ml of 30% sucrose under 0.6 ml 15% sucrose. Both sucrose layers contained 2.0 M NaCl, 1.0 mM EDTA, 10 mM Tris, pH 8.0. The gradients were spun at 4°C in a microfuge for 4 min at 9000 g. The nucleoids formed a white aggregate that usually sedimented to the interface between the two layers of sucrose. These nucleoids were recovered in a volume ranging from 200 to 300 μl and a small aliquot was stained with ethidium bromide 80 $\mu\text{g}/\text{ml}$ and examined by fluorescence microscopy for assessing integrity and DNA-loop supercoiling (24). The recovered nucleoid suspension was washed in 14 ml of PBS-A at 4°C for 4 min at 1500 g (34). The fluid pellet was recovered in a volume ranging from 200 to 300 μl .

DNase I digestion of nucleoid samples

The washed nucleoids are pooled for setting up the DNase I digestion curves (2×10^6 nucleoids in 1.2 ml of PBS-A) and mixed with DNase I digestion buffer: 10 mM MgCl_2 , 0.1 mM dithiothreitol, 50 mM Tris at pH 7.2 (usually 5 ml of digestion buffer for each 1.2 ml of nucleoid suspension). Digestions were carried out at 37°C with 0.5 U/ml DNase I (Sigma). Each digestion time point aliquot contains 3.5×10^5 nucleoids. Digestion reactions were stopped by adding enough stop buffer to achieve an EDTA concentration of 30 mM. The stop buffer contained 0.2 M EDTA and 10 mM Tris at pH 7.5.

Washing protocol for preparing NM-bound DNA as template for PCR

After digestion with DNase I, the nucleoid samples were further handled as previously described (34), briefly: stirred in the vortex for 15 s and then centrifuged for 10 min at 9000 g at 4°C. The nucleoid pellet was mixed with 1 ml PBS-A, stirred, and centrifuged again as above. The pellet was mixed with 1 ml double-distilled (dd)- H_2O , stirred, and centrifuged as above. The last step was repeated twice. The final pellet was resuspended in dd- H_2O (100–200 μl) to be used directly as template for PCR.

PCR amplification

NM-bound DNA (400 ng) was used as template for PCR. Standard PCR was carried out using Taq Polymerase (GIBCO-BRL) in an Applied Biosystems GeneAmp PCR System 2400 thermocycler. Following the appropriate controls previously described (34), the same amplification programme was used for all pairs of primers (25 pmol): initial denaturation step at 94°C for 5 min, denaturation step at 94°C for 45 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min for 35 cycles, with a final extension at 72°C for 10 min. The identity of all the amplified sequences was confirmed by restriction analysis with the appropriate restriction enzymes. Amplified PCR products were electrophoresed on 2% agarose gels and visualised using ethidium bromide staining, recorded and analysed using a Kodak 1D Image Analysis Software 3.5 system.

Genomic DNA primers

For the positional mapping of specific gene sequences relative to the nuclear matrix we used the following primers.

Rat β -actin (accession nos V01217, J00691): sense primer (Sen), 5'-CGTAAAGACCTCTATGCCA; antisense primer (Ant), 5'-AGCCATGCCAAATGTGTCAT; amplicon size, 473 bp; location, exon 5–intron 5–exon 6, chromosome 12q11.

Universal β -actin (accession no. M10277): Sen, 5'-AAC-ACCCCAGCCATGTACG; Ant, 5'-ATGTCACGCACGA-TTTCCC; amplicon size, 254 bp; location, exon 4, chromosome: 12q11.

Rat albumin gene (accession no. M16825): Sen, 5'-GGGATTTAGTTAAACAACCTT; Ant, 5'-AAAGGTTACC-CACTTCATTG; amplicon size, 206 bp; location, promoter/exon1, chromosome 14p22.

Rat alpha-fetoprotein gene (accession no. J02816): Sen, 5'-ACCCATGCATCTGTGACATA; Ant, 5'-AGTAAAATGC-ATGTTGCCTG; amplicon size, 252 bp; location, 5' flanking region, chromosome 14p21.

Rat *c-myc* gene (accession no. Y00396): Sen, 5'-TAT-AATCCGGGGGTCTGCGC; Ant, 5'-CCCTCTGTCTCTC-GCTGGAA; amplicon size, 227 bp; location, untranslated promoter, chromosome 7q33.

Rat collagen type I alpha-1 gene (accession no. J04464): Sen, 5'-CATACCTGGGCCACACCAT; Ant, 5'-CTTGCA-CTTTCCTTCTGGGA; amplicon size, 261 bp; location, 5' flanking region, chromosome 11.

Rat inositol trisphosphate receptor subtype 3 (*IP3R3*) mRNA (complete cDNAs, accession no. L06096): Sen, 5'-GGTGAGCGGCGAGGGCGAGG; Ant, 5'-GCAGTTCTG-CACGTCCACGA; amplicon size, 480 bp; location, nucleotides 7961–8136 in mRNA, chromosome 20.

RT-PCR

Total RNA of isolated hepatocytes was obtained using RNeasyTM-4PCR (Ambion). All RNA samples were quantified and normalised using spectrophotometric methods. Double-stranded cDNA was synthesised from extracted RNA using SUPERScript II (GIBCO-BRL) and random primers according to the manufacturer's protocol. Reverse transcription was performed using 500 ng of RNA. The PCR primers chosen were complementary to sequences in the mRNA. Different amplification programmes were used depending on the pair of primers (25 pmol): initial denaturalisation step at 94°C for 5 min, denaturalisation step at 94°C for 45 s, annealing at 56–60°C for 30 s, and extension at 72°C for 1 min for 30–32 cycles, with a final extension at 72°C for 10 min (specifically: β -actin, 56°C 30 cycles; *IP3R3*, 56°C 32 cycles; *ALB*, 58°C 30 cycles; *AFP*, 60°C 32 cycles; *c-myc*, 60°C 30 cycles; *COL1A1*, 60°C 30 cycles).

RT primers

Rat gene encoding cytoplasmic β -actin (accession nos V01217, J00691): Sen, 5'-CGTAAAGACCTCTATGCCAA; Ant, 5'-AGCCATGCCAAATGTGTCAT; amplicon size, 349 bp; location, exon 5–exon 6.

Rat inositol trisphosphate receptor subtype 3 (*IP3R3*) mRNA (complete cDNAs accession no. L06096): Sen, 5'-GGTGAGCGGCGAGGGCGAGG; Ant, 5'-GCAGTTCTG-

CACGTCCACGA; amplicon size, 175 bp; location, nucleotide 7961–8136 in mRNA.

Rat albumin (*ALB*) mRNA (accession no. NM_134326): Sen, 5'-ATACACCCAGAAAGCACCTC; Ant, 5'-CAC-GAATTGTGCGAATGTGTCAC; amplicon size, 436 bp; location, nucleotide 1301–1737 in mRNA.

Rat alpha-fetoprotein (*AFP*) mRNA (accession no. NM_012493): Sen, 5'-CAGTGAGGAGAAACGGTCCG; Ant, 5'-ATGGTCTGTAGGGCTCGGCC; amplicon size, 252 bp; location, nucleotide 1421–1673 in mRNA.

Rat v-myc avian myelocytomatosis viral oncogene homologue (*c-myc*) mRNA (accession no. NM_012603): Sen, 5'-AGTGCATTGATCCCTCAGTGGTCTTTCCCTA; Ant, 5'-CAGCTCGGTTCTCTCTGACGTTCCAAGA-CGTT; amplicon size, 548 bp; location, nucleotide 976–1524 in mRNA.

Rat collagen type I alpha-1 gene (*COL1A1*) exon 1 (accession nos XM_109776, J04464): Sen, 5'-GAGT-GAGGCCACGCATCAGCCGAAGCTAAC; Ant, 5'-AAG-AGGAGCAGGAGCCGGAGGTCCACAAAG; amplicon size, 111 bp; location, nucleotide 33–144 in mRNA.

RESULTS

A protocol for mapping gene positions relative to the nuclear substructure

The DNA loops plus the nuclear substructure constitute a nucleoid. The nuclear substructure present in nucleoids isolated as described in Materials and Methods was originally named nuclear cage (1). Yet, for the sake of simplicity we shall employ the more common term nuclear matrix (NM) when referring to the nuclear substructure present in our nucleoid preparations. Under the conditions of lysis employed to generate nucleoids, the DNA remains essentially intact, although it lacks the nucleosome structure because of the dissociation of histones and most other nuclear proteins usually associated with DNA; yet, the DNA loops remain topologically constrained and supercoiled (1,2). Nucleoids prepared from freshly isolated hepatocytes from either control or hepatectomised rats were subjected to the protocol outlined (Fig. 1A) in order to map the relative position of specific gene sequences to the nuclear substructure. Our method involves the progressive detachment of DNA from the nuclear substructure by digestion with a carefully adjusted concentration of a non-specific endonuclease (DNase I), followed by specific procedures to remove any DNA fragments detached from the NM and then carrying direct PCR-amplification of specific target sequences on NM-bound templates (34). It is well established that resistance to nuclease-mediated detachment is directly proportional to the DNA sequence proximity to the anchoring point on the NM. This is probably due on the one hand to the phenomenon of steric hindrance (17,37,38), whereby DNA sequences located close to the NM are relatively protected from endonuclease action by being immersed in the matrix framework that may act as a physical obstacle to any incoming macromolecule in solution. On the other hand, the stochastic action of non-specific nucleases such as DNase I causes random cutting on loop DNA in such a way that it will release faster, on average, sequences located in a more distal position relative to the nuclear substructure.

Figure 1. (A) Outline of the general protocol for mapping specific gene positions relative to the NM by PCR-amplification of the target sequences on NM-bound templates. (B) Freshly isolated hepatocytes. (C) Nucleoid obtained from an isolated hepatocyte and stained with 80 µg/ml of ethidium bromide, note the homogeneous halo of DNA surrounding the NM. The inset shows the same NM observed by phase-contrast microscopy. (D) Nucleoid thoroughly digested with DNase I and incubated with anti-lamin B1 antibody and further stained with a rhodamine-linked secondary antibody, showing the perimeter of the residual nuclear substructure. $\times 40$ objective in all micrographs.

Therefore, for the mapping protocol to work it is necessary that the loop DNA is equally accessible to the endonuclease (except for those regions close to the nuclear substructure framework). This condition is achieved by high-salt extraction during nucleoid preparation and by using a non-specific nuclease, as is the case in our protocol.

The kinetics of nucleoid-DNA digestion correlate with the replicating status of the hepatocytes

Surgical ablation of $\sim 70\%$ of the liver mass leads to massive re-entry into the cell cycle of some 95% of the residual hepatocytes, this is followed by a well characterised first large peak of DNA synthesis at 24 h, followed by a smaller peak between 36 and 48 h (32,35,39). Restoration of the original number of hepatocytes requires 1.6 proliferative cycles per residual hepatocyte, therefore most hepatocytes participate in one or two proliferative events and then return to the G0 phase of the cell cycle. The whole regeneration process usually lasts 7 days after which there is full recovery of the original cell mass and liver function (for reviews see 32,33). Figure 2 shows the kinetics of DNA digestion with a low concentration of DNase I (0.5 U/ml) of hepatocyte nucleoids from control and regenerating livers at different times after partial hepatectomy. The digestion curves provide the basic information for calibrating the positional mapping of specific DNA sequences to the NM, since aliquots of each digestion time-point are used for PCR amplification of the selected sequences on NM-bound templates. Control and samples from 2 h and 7 days PH show a very similar amount of residual DNA attached to the NM after 60 min digestion. Yet, samples from 24 and

48 h PH show faster kinetics of digestion: the residual DNA attached to the matrix after 60 min of digestion is less than half the value of the control sample. This indicates greater sensitivity of DNA to endonuclease digestion in nucleoid samples from actively proliferating hepatocytes. This phenomenon has been previously described in nucleoids from actively growing cells (25,40), and is readily explained by the discontinuous model of DNA synthesis because on one side of the replication fork the DNA is single-stranded (25,41,42), such single-stranded DNA is more easily cleaved by DNase I, which introduces single-stranded nicks into the DNA (43). However, nucleoid samples from 96 h PH are significantly more resistant to DNase I digestion than the control. The samples resistant to DNase I digestion correspond to cells at the well known 'overshoot' of hepatic energy status that occurs between 72 and 120 h PH, which coincides with actual cessation of cell proliferation and re-entry of hepatocytes into G0 (35,39,44).

Genes become closer to the NM at the peak of DNA synthesis after partial hepatectomy

We chose specific sequences belonging to six different genes for mapping their relative position to the NM. Four genes: β -actin, *c-myc*, collagen type 1 (*COL1A1*) and inositol 1,4,5-trisphosphate receptor type 3 (*IP3R3*), are located in four different rat chromosomes (see Materials and Methods), while two other genes: albumin (*ALB*) and alpha-fetoprotein (*AFP*), are located within 15 kb of each other in the multi-locus region of the albumin-gene family cluster in the long arm of chromosome 14 of the rat (45). The β -actin gene is an example of a constitutive gene expressed in most rat tissues since β -actin protein is a major structural constituent of most cell types. The gene *c-myc* is poorly expressed in quiescent hepatocytes but it quickly increases its transcription within 2 h of partial hepatectomy in both rat and mouse liver (33,46,47). *COL1A1* shows enhanced expression within 12 h of partial hepatectomy (48). *IP3R3* was initially chosen as an example of a non-expressed gene in the liver (49). *ALB* is a highly expressed gene in rat adult liver, since albumin is the most abundant protein in serum, while *AFP* is reversibly repressed in adult liver and becomes active in the course of liver regeneration after partial hepatectomy (45,50). Figures 3 and 4 show the specific amplicons corresponding to the chosen sequences of the specified genes. As usual, the particular efficiency of amplification varies among the different amplicons studied, depending on the amplicon length and specific target sequence (34). However, it must be stressed that all amplicons tested were within the 480–206 bp range and the same amplification programme was used for all pairs of primers (Materials and Methods), including 35 cycles and the fact that equal amounts of NM-bound DNA were loaded in the control and partially digested PCRs. All these factors favour the amplification of low-efficiency templates. Therefore, we may be confident that the extinction of a target amplicon at a given DNA-digestion time represents the actual reduction of the corresponding sequence to a non-amplifiable level (within the nucleoid population analysed) and not the lack of enough template DNA to carry out the PCR.

The amplicon signals are scored as positive or negative, based on whether they are detected or not by a digital image analysis system (Materials and Methods). For example, the

Figure 2. Kinetics of DNA digestion in nucleoids from regenerating hepatocytes at different times PH. Nucleoid samples were digested with DNase I (0.5 U/ml) and further processed as described in Materials and Methods. The residual amount of NM-bound DNA was quantified by spectrometry. Each time-point value is the average of separate DNA digestion curves of nucleoids obtained from independent animals. Non-hepatectomised control (CT), $n = 6$ rats. PH samples taken at 2, 24, 48, 96 h and 7 days, $n = 3$ rats for each DNA digestion curve corresponding to each particular time PH.

β -actin amplicon is observed in samples from control hepatocyte nucleoids at 0, 5 and 15 min of DNA digestion, but it is absent in samples digested for 30 and 60 min. However, in nucleoids of hepatocytes isolated from livers at 24 h PH the β -actin amplicon is detected up to 30 min of DNA digestion, suggesting that at this stage the β -actin gene becomes positioned relatively closer to the NM. At 48 h PH the amplicon is no longer detected in samples digested for 30 min (Fig. 3), suggesting that the gene is regaining its original position relative to the NM.

However, the PCR amplification results need to be calibrated using as a gauge the percentage of total DNA remaining associated with the NM at each digestion time and according to the stage-specific kinetics of nucleoid-DNA digestion shown in Figures 2 and 4A and C. Thus, Figure 5 presents this kind of analysis for all the amplifications displayed in Figures 3 and 4. The bars represent 'positional windows' of total DNA associated with the NM within which it is highly probable, on average, to find the mapped genes. The lower number in each positional window corresponds to the percentage of total DNA bound to the NM at which the amplification of the specific target sequence is certainly extinguished. The β -actin gene becomes progressively closer to the NM after hepatectomy, reaching its closest position at 24 h PH, but then gradually becomes further removed from the NM and eventually relocates itself to a positional window similar to the control. In the control hepatocytes the β -actin

gene is located somewhere within the 41–27% of total DNA closest to the NM, while at 24 h PH the same gene is located within the 11–10% of total DNA closest to the NM, and it becomes located within 55–28% of the total DNA closest to the NM at 7 days PH when the liver regeneration process is basically over (Fig. 5). Interestingly, *IP3R3*, a supposedly non-expressed gene in the liver, reaches its furthest position from the NM at 2 h PH, something not shown by any of the other five genes mapped and previously known to be expressed in the liver (Fig. 5). Indeed, all genes mapped reach their closest position relative to the NM at 24 h PH (Fig. 5), this coincides with the maximum peak of DNA synthesis characteristic of liver regeneration after partial hepatectomy (32,33,35). At 48 h PH most genes become distal to the NM, except *AFP*, which remains at the 24 h PH position, while at 96 h PH all genes mapped are located further away from the NM than in the control. Yet, by 7 days PH all genes relocate to a positional window similar or compatible with the control (Fig. 5).

Further mapping experiments suggest transient relocation of genes to a position furthest from the NM when cells re-enter G0

Considering the different kinetics of nucleoid-DNA digestion observed at different times PH (Fig. 2), it was necessary to carry out further mapping experiments for *AFP* in such a way that the gene positional window at 2 h PH could be clearly

Figure 3. Specific PCR-amplification products corresponding to the target sequences of the six genes (β -actin, *IP3R3*, *ALB*, *AFP*, *c-myc* and *COL1A1*) whose position relative to NM was mapped in nucleoids from regenerating hepatocytes PH. Nucleoids from isolated hepatocytes were treated with DNase I (0.5 U/ml) for 0, 5, 15, 30 and 60 min and further processed for direct PCR amplification of the target sequences on NM-bound templates (see Materials and Methods). The amplicons were visualised in 2% agarose gels stained with ethidium bromide. The presence of the target sequence in the residual NM-bound DNA of the partially digested samples was scored as positive (independently of the actual intensity of the amplicon band) if the amplicon was detected by the Kodak 1D Image Analysis Software version 3.5. CT, non-hepatectomised control; PH samples were taken at 2, 24, 48, 96 h and 7 days. The amplicon patterns were consistently reproduced in separate experiments with samples from independent animals (CT, $n = 6$ rats; PH time-points, $n = 3$ rats each).

Figure 4. Further mapping of the *AFP* and *ALB* gene positions relative to the NM at 2 h (*AFP*) and 96 h (*AFP*, *ALB*) PH. (A) Long-term kinetics of DNA digestion (120 min) in nucleoids from regenerating hepatocytes at 2 h PH. (B) *AFP* amplicon detected up to 60 min but not at 90 min post-DNase I digestion in nucleoid samples from rat hepatocytes at 2 h PH. (C) Long-term kinetics of DNA digestion in nucleoid samples from regenerating hepatocytes at 96 h PH. Notice the slower kinetics of DNA digestion at 96 h PH and the larger amount of residual NM-bound DNA after 120 min of DNase I digestion (2 h = 15.2%; 96 h = 27.8%). (D) *ALB* amplicon detected at 60 min but not at 90 min post DNase I digestion in nucleoid samples from rat hepatocytes at 96 h PH. (E) *AFP* amplicon detected at 90 min but not at 120 min post-DNase I digestion in nucleoids from rat hepatocytes at 96 h PH. All DNA digestions with DNase I at 0.5 U/ml. M, MW DNA ladder = 50 bp (Gibco-BRL).

Figure 5. Positional windows relative to the NM for each gene mapped in nucleoid samples at different times PH (2, 24, 48, 96 h and 7 days). The upper values on each bar correspond to percentage of total DNA associated with the NM at which each specific amplicon was last detected. The lower values correspond to percentage of total DNA associated with the NM at which each specific amplicon was no longer detected. Hence, the specific DNA sequence corresponding to each amplicon is expected to be actually located within the interval defined by such values. The nucleoid-DNA digestion curves depicted in Figure 2 were used as gauges for establishing most of such intervals based on the amplification data shown in Figure 3. The bars marked with asterisks were obtained using as gauges the nucleoid-DNA digestion curves depicted in Figure 4 and the corresponding amplification data shown in Figures 2 and 4.

distinguished from the positional window at 24 h PH. The results show that *AFP* is located within the 22–20% of total DNA at 2 h PH (Figs 4A and B and 5), while at 24 h PH it is located within the 10% of total DNA closest to the NM (Figs 2, 3 and 5). Further mappings were also carried out for *AFP* and *ALB* at 96 h PH. The results show that *AFP* is located within 38–28% of total DNA at 96 h PH, but within the 10.5% of total DNA closest to the NM at 48 h PH (Figs 2, 3, 4C and E and 5). *ALB* is located within 42–38% of total DNA at 96 h PH, but it is within 22–10.5% of total DNA at 48 h PH (Figs 2, 3, 4C and D and 5). It is worth noting the relative resistance to DNase I digestion shown by DNA from nucleoids corresponding to 96 h PH (Figs 2 and 4C), suggesting that a major modification of higher-order DNA structure occurs when the cells re-enter into G0 (35,39,44). Yet, this seems to be a transient phenomenon, since by 7 days PH the kinetics of nucleoid-DNA digestion is similar to the one observed in non-hepatectomised controls (Fig. 2) and this corresponds to the stage PH when liver mass and function are considered as fully recovered (32,33).

In primary hepatocytes there is no correlation between a gene position relative to the NM and the gene-expression level

Many reports indicate a correlation between proximal position or actual attachment of a given gene to the nuclear substructure and its transcription (11–16). Thus, we performed standard RT-PCR experiments in order to assess the expression of the six genes mapped. After partial hepatectomy both

the *c-myc* and *COL1A1* genes display a significant increase in their expression (Fig. 6), this being consistent with previous reports (46,48). However, *c-myc*, a typical example of an immediate early gene expressed PH, shows enhanced expression that peaks at 2 h PH, before reaching its closest position to the NM at 24 h PH. On the other hand, *COL1A1* shows progressive enhanced expression that peaks at 48 h PH and then levels off or gradually declines (Fig. 6). Indeed, most genes whose expression is stimulated by hepatectomy return to their basal expression values within 14 days PH (32,51,52). Thus, although the *COL1A1* expression pattern observed PH somehow correlates with the positional changes of this gene relative to the NM (Figs 5 and 6), all other genes display expression patterns that cannot be correlated to their actual relative position to the NM at any particular PH stage. We observed no significant difference in the expression pattern of β -actin, *ALB* and *AFP* within 7 days PH (Fig. 6), suggesting a constant level of expression for these genes during the liver regeneration process. This fact is not surprising for the case of β -actin and *ALB*. However, the *AFP* gene is regarded as being repressed reversibly in the adult rat liver, since its strong expression in the embryonic period is basically switched off after birth (45), although it is possible to detect a very slight basal expression of *AFP* in the adult liver (53; Fig. 6). Yet, there are reports of a moderate rise in *AFP* mRNA during the first 4 days following partial hepatectomy in rats (54). The *IP3R3* gene that we chose as an example of a non-expressed gene in the liver (49) shows a slight, fluctuating but

Figure 6. Expression of the six genes positionally mapped relative to the NM, monitored by RT-PCR as described in Materials and Methods. The specific amplicons of the target cDNA sequences and their corresponding expression patterns (2, 24, 48, 96 h and 7 days) PH are shown. Each time-point value in each expression curve corresponds to the average of three independent determinations in samples from different animals similarly treated ($n = 3$ rats for each time point). M, MW DNA ladder = 50 bp (Gibco-BRL).

measurable expression pattern, being detectable in both control and PH samples (Fig. 6). This was independently confirmed by another group (A.Martínez-Gómez and M.A.R.Dent, personal communication).

Mapping of two regions within the β -actin gene confirms that the gene becomes closer to the NM when the hepatocytes shift from quiescence to proliferation

The constitutive β -actin gene shows a constant level of expression throughout the processes of liver regeneration after partial hepatectomy (Fig. 6). Therefore, this gene could be used as a probe to explore whether the gene positional changes relative to the nuclear substructure are indeed correlated to the proliferating status of the hepatocytes. Two different regions located around the middle of the β -actin gene but set 320 bp apart from each other (Fig. 7A), were used as targets for amplification in NM-bound templates from control and PH samples. Figure 7B shows that the X sequence (universal actin, 254 bp in length) located closer to the 5'-end of the β -actin gene, amplifies in control samples digested up to 30 min with DNase I, but is negative in samples digested for 60 min. This locates the X sequence within the 27.3–25.8% of total DNA closer to the NM according to the corresponding kinetics of nucleoid-DNA digestion (Fig. 2). However, the same sequence mapped in nucleoids from 24 h PH, still amplifies in samples digested for 60 min, which means that the X sequence is now located within the 10.4% of total DNA closest to the NM (see the corresponding kinetics in Fig. 2). This is similar to the closest positional window relative to the NM shown by the AFP gene at 24 h PH (Fig. 5). On the other hand, the Y sequence (the previously mapped rat-actin amplicon of 473 bp) located closer to the 3'-end of the β -actin gene, maps in the control samples within 41.1–27.3% of total DNA closest to the NM (Figs 2, 5 and 7B), while in the 24 h PH samples it moves to a position within 11.2–10.4% of total DNA closest to the NM (Figs 2 and 7B). The simplest interpretation of these results is shown in Figure 7C. Thus, at least in the case of β -actin, the gene seems to be orientated with its 5'-end towards the DNA loop anchoring point to the NM, and the results suggest that the whole gene becomes

closer to the NM when the hepatocyte shifts from a quiescent to a proliferating state.

DISCUSSION

We have mapped in freshly prepared hepatocytes the position relative to the nuclear substructure of six different genes. In nucleoids, the relative resistance of a given DNA sequence to detachment from the NM by a non-specific nuclease is just a statistical phenomenon resulting from the particular position of the sequence relative to the NM attachment point, and it reflects no intrinsic resistance of the particular DNA sequence to the nuclease. In a large nucleoid sample a sequence close to the NM is less likely to be digested within the first minutes of DNase I treatment. Therefore, the observed degree of resistance to digestion of a given DNA sequence corresponds to its average across the nucleoid population analysed and the positional mapping results represent the most probable average location relative to the NM. Moreover, a gene can resist detachment from the NM because it actually lies close to the point of attachment in every cell or because it does so in a large fraction of the cell population studied; we cannot formally rule out either of these two possibilities. Thus, we are actually monitoring the average DNA loop arrangement in a large number of hepatocytes. Therefore, our mapping approach must be regarded as semi-quantitative and topological (non-metric).

In the case of four genes: ALB, AFP, c-myc and COL1A1, we chose sequences corresponding to the 5'-ends of such genes as targets for PCR amplification. In quiescent hepatocytes all four genes lay within a narrow positional window corresponding to 27–25% of the total DNA closest to the NM (Fig. 5). For β -actin and IP3R3, we chose sequences located somewhere between the middle and 3'-ends as targets. In quiescent hepatocytes both genes mapped within 41.1–27.3% of the total DNA closest to the NM. Considering that the six genes mapped are located in five different rat chromosomes and thus represent different territories or compartments within the rat hepatocyte nucleus (29,31, 55,56), our results suggest that in quiescent (G0) cells there is a trend for most genes to be located within a rather narrow

Figure 7. Positional mapping relative to the NM of two separate regions within the β -actin gene. (A) The location of the X sequence (universal actin, 254 bp in length) and the Y sequence (rat-actin amplicon, 473 bp in length) are shown on the β -actin gene. The sequences are set 320 bp apart from each other. (B) Specific amplicons of the target sequences in nucleoids from control (CT) and hepatocytes from 24 h PH digested for different times (0–60 min) with DNase I (0.5 U/ml). The positional windows were established using as gauge the corresponding DNA digestion curves depicted in Figure 2 (see text for details). (C) Diagram deduced from the results of this type of experiment, showing the location of both X and Y sequences within the corresponding DNA loop in quiescent (CT) and proliferating (24 h PH) hepatocytes. The thick basal line represents the NM (diagram not scaled).

positional window relative to the NM, a suggestion reinforced by further results (Fig. 7), since when mapping a second sequence that also belongs to the β -actin gene but is located some 574 bases closer to the 5'-end of the gene, the position of the β -actin gene relative to the NM shifts from 41.1–27.3% to 27.3–25.8% of total DNA closest to the NM which is basically the same positional window of the genes that were mapped using 5'-end sequences as targets. The average DNA-loop size in mammalian cells is 86 kb (57). Thus, the fully extended length of the DNA loop from the anchoring points at the NM to the most distal tip of the loop would be ~43 kb. The β -actin gene is 4.1 kb in length, thus it easily fits within a single DNA-loop arm. In quiescent hepatocytes, the Y sequence of the β -actin gene lies within a positional window whose lower boundary corresponds to 27.3% of total DNA closest to the NM, while the lower boundary of the positional window for the X sequence corresponds to 25.8% of total DNA closest to the NM. The 5'-end of the Y sequence is 574 bp distant from the 5'-end of the X sequence (Fig. 7A), this roughly equals 1.3% of the average DNA loop length, the value of which is very close to the 1.5% difference between the lower boundaries of the Y and X positional windows. Moreover, 27.3% of total DNA-loop length equals 11.7 kb, while the 25.8% equals 11 kb; the 0.7 kb difference is very close to the 574 bp that sets apart the 5'-ends of the X and Y sequences.

In synchronised cells newly replicated DNA is bound to the NM (25,58–60). DNA replication 'factories' have been visualised as discrete foci organised upon the nuclear substructure (21,61,62). Indeed, location and distribution of replication sites is predetermined independently of the actual proliferating status of the cell (63). It is known that there is a correlation between the average size of DNA loops and the average size of replicons in different types of eukaryotic cells (64), and it seems that replication origins are located in or around permanent sites of DNA attachment to the NM (6–8,65). Topological considerations concerning the problems posed by tracking polymerases moving upon a structurally complex template such as DNA, support a reeling model for DNA replication according to which the DNA reels through the replication factory that extrudes newly replicated DNA (21,42). Replication factories are large molecular complexes where several DNA loops may fit in at the same time (21). Thus, in the S phase the gene sequences located in such loops may be found closer to the nuclear substructure because they are moving towards or away from the replication factories. Therefore, the larger the population of cells in S phase, the greater the probability of finding early-replicating sequences proximal to the nuclear matrix. Our results show that at 24 h PH, when there is maximal DNA synthesis in the regenerating liver, all six genes mapped are found within the 11% of total

DNA closest to the NM, and this is consistent with the genes either getting in or coming out from the fixed replication factories on the NM.

Usually gene-rich chromosome regions tend to replicate early in S phase, while the gene-poor, non-coding or heterochromatin regions are replicated late in S phase (66). Our present and previous results (34) suggest that all genes, whatever their expression status, are located more or less at about the same distance from the NM (corresponding in quiescent hepatocytes to the 28–25% of total DNA closest to the NM; Fig. 5). Moreover, very detailed positional mappings indicate that the positional-window difference between an actively expressed and a non-expressed gene could be as slight as 1–2% of total DNA closer to the NM (34). This is consistent with the fact that, on the whole, genes tend to replicate earlier than non-coding DNA.

At 96 h PH, when cells re-enter G₀, most genes reach their furthest position relative to the NM, but then by 7 days PH, when the liver regeneration is basically completed and function has been restored, the genes regain their original position. These movements suggest a process of gradual adjustment of gene positioning relative to the NM after mitosis. In recent years several reports have indicated that chromatin moves locally as well as on a large scale within the interphase nucleus (28,67–70). Large-scale chromatin movements in early G₁ correlate with the repositioning of sub-chromosomal regions within positionally stable territories, and this includes small-scale refolding events within sub-chromosomal regions (68,71,72). Such local chromatin movements within larger, rather stable territories might lead to DNA–protein and protein–protein interactions that mediate the attachment or detachment of DNA sequences to and from the NM. The mitotic chromosomes are organised in condensed DNA loops anchored to a chromosome scaffold that appears to share some constitutive elements with the NM (30,73–76). Thus, the NM should be reassembled, at least in part, from the chromosome scaffolds after mitosis.

However, restoration of nuclear architecture and topology typical of the interphase nucleus may not be immediate. Early G₁ or early G₀ might correspond to stages of relative instability in the interactions between DNA and the NM. Specific DNA-affinity sites in the NM can detain a subset of sequences present in total DNA that corresponds to the MARs. (77), but not all MARs co-localise with the actual DNA loop anchorage sites (78,79). It has been suggested that within the genome there is a range of non-coding DNA sequences with various degrees of affinity for attachment sites at the NM. The high-affinity DNA sequences form the structural (permanent) attachments to the NM, but after mitosis, in early G₁, the actual pattern of DNA attachments to the NM characteristic of the cell type and state of differentiation shall be re-established by random scanning between potential MARs and actual attachment sites available at the NM (80). This is consistent with the observed relative resistance to DNase I shown by DNA from nucleoids at 96 h PH, suggesting that a larger proportion of the total DNA is transiently closer to the NM, something that may occur if there is a larger number of attachments of DNA to the NM resulting in shorter than average DNA loops. Yet, the probability of spurious attachments occurring in non-coding regions far from any gene is larger than the probability of spurious attachment occurring in

a sequence that is close to a gene, because there are more gene-empty than gene-dense areas. Indeed, there is evidence that attachments to the nuclear substructure are on average twice as frequent in the gene-poor regions as in the gene-rich regions of the genome (81). Thus, most genes will temporarily appear to be further from the NM though they will show no particular preference to be located within a narrow positional window common to all genes (as is the case at 96 h PH, see Fig. 5). However, the topological order within the nucleus must be re-established, otherwise the cell will not be viable anymore (24,26). This correlates with the fact that in the quiescent condition (G₀) the genes are located within a narrow positional window relative to the NM (Fig. 5).

Previous reports have suggested that attachment to the NM is characteristic of active genes (11–16). However, the RT-PCR experiments (Fig. 6) coupled to the positional mapping results suggest that genes are positioned relative to the NM in a way that is independent of their transcriptional status. The correct estimation of a distance between a sequence of interest and a DNA-loop anchorage site depends on the equal accessibility of the whole DNA loop (except for those sequences attached or very close to the NM), to the nuclease used for the detachment of DNA from the NM (17). This condition was disregarded in previous mapping protocols by the use of sequence-specific restriction endonucleases instead of a general nuclease (such as DNase I) a fact that undermines their resolution. Moreover, the so-called temporal or facultative attachments to the NM are sensitive to extraction with low ionic strength while the permanent, structural attachments are not (82). Thus our high-salt extraction procedure would not distinguish between temporal or permanent attachments but simply between high-salt resistant and non-resistant attachments. It has already been pointed out that extraction with high salt reveals sequences with some of the global properties that sample components of both an internal chromosome scaffold and an external NM (81). Thus, in our hands, transcription-mediated attachments to the NM, if any, appear to be non-resistant to high-salt extraction.

The present work shows that changes in the topological relationships between the DNA and the nuclear substructure correlate with the actual proliferating status of cells in a living tissue (the liver), and suggests that specific modifications in higher-order DNA structure are characteristic of the quiescent (G₀) and replicating (S) phases of the cell cycle *in vivo*.

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