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Activation of the lamin A gene during rat liver development

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Abstract **We have studied the regulation of expression of the A-type lamins, which are constituents of the nuclear lamina. During rat liver development, high levels of lamin A and C mRNAs were observed in 15-day fetal liver but were barely detectable in the adult. The chromatin conformation of the lamin A gene was sensitive to DNase I in 15-day fetal liver but became mostly insensitive in the adult. Lamin A and C proteins could be detected in fetal liver and persisted in the adult. Our evidence suggests that the lamin A gene is actively transcribed early in liver differentiation and its activity declines considerably in adult liver.**

Key words: Lamin A gene; Nuclear lamina; Liver development

1. Introduction

The lamins are components of the nuclear lamina, a filamentous network underlying the inner nuclear membrane on the nucleoplasmic face. The lamins play a crucial role in nuclear disassembly and reassembly during cell division and have been classified as a subtype of the intermediate filament family of proteins [1–3]. In higher vertebrates, the lamins are of two kinds: A-type and B-type. During mitosis, the B-type lamins (B1 and B2) remain associated with nuclear envelope vesicles, whereas the A-type lamins (A and C) become solubilized [4]. In mammals, birds and amphibians, B-type lamins are expressed in all somatic cells, whereas the expression of lamins A and C is detectable only at later stages of development [5–8]. Immunocytochemical studies have revealed that undifferentiated mouse embryonal carcinoma cell lines and early mouse embryos contain only lamin B, whereas lamins A and C are expressed later in embryogenesis, when tissue differentiation is initiated [5,6]. Lamins A and C have identical amino acid sequences except for their C-terminal domains, and most probably arise by differential splicing of RNA from a single lamin A gene [3,9,10]. Although structure-function correlations of the lamins have been examined in detail, there have been relatively few studies on the transcriptional status of the lamin genes [11,12]. The mechanisms contributing to lamin gene regulation during mammalian embryonic development or the functional significance of its differential expression are not known.

A convenient tissue model for the study of developmental changes in gene regulation in the rat or mouse is the liver. The ontogeny of expression of two liver-specific proteins, albumin and α-fetoprotein, has been studied in detail. A parallel accumulation of albumin and α-fetoprotein RNAs is observed early in development, followed by a selective decrease in α-fetoprotein RNA at birth [13]. The regulation of these genes occurs primarily at the level of transcription. This differential gene expression in developing liver provides sufficient background for the study of regulatory mechanisms of other developmentally activated genes such as the A-type lamins.

In order to understand how the lamin A gene is regulated, we have initially assessed lamin A expression during rat liver development by Northern analysis of RNA and nuclear run-on transcription assays. The unfolding of the chromatin structure of the gene has been studied and lamin A protein levels determined. Our evidence suggests that the lamin A gene is actively transcribed by day 15 of embryonic liver development.

2. Materials and methods

2.1. Maintenance of cell lines

The rat fibroblast cell line, F-111, and mouse embryonal cell line, PCC-4, were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were harvested for RNA isolation at 60–80% confluency.

2.2. Isolation of nuclei and nuclear envelopes

Livers were dissected from Wistar rats at different stages of development, and processed to obtain nuclei by sucrose density centrifugation over 2.2 M sucrose to preferentially pellet nuclei and exclude whole cells [14]. Purified nuclei were digested with nucleases and salt-extracted as described [15] to yield nuclear envelopes. The absence of extranuclear contaminants and intranuclear material in the envelopes was verified by marker analysis as described earlier [16]. The homogeneity of the nuclear envelope preparation was confirmed by phase contrast microscopy, wherein the envelopes were seen as nucleus-sized vesicles [16].

2.3. Nucleic acid probes

The rat lamin A cDNA clone, prlamA, was isolated from a rat liver lambda ZAPII cDNA library and contains a nearly full length insert (2.9 kb) cloned into the pBS vector [11]. The plasmid pmalb2 contains a 700 bp insert of mouse albumin cDNA in pBR322; pmAFP1 contains a 900 bp insert of mouse α-fetoprotein cDNA in pBR322; pHh4A-SP3 contains a 840 bp insert of the human H4 gene in pSP65; β2000 contains a 2.1 kb insert of the chicken β-actin gene in pBR322.

2.4. Antibodies, immunoblot analysis

Antibodies were raised to bacterially expressed rat lamin A, obtained as a glutathione-*S*-transferase fusion protein. The plasmid construct used contained a 2.9 kb *Eco*RI fragment of the prlamA gene [11] cloned into the pGEX-2T vector [17]. Upon induction with isopropyl thiogalactoside, a fusion protein of 100 kDa was obtained as expected, purified by electroelution from SDS-polyacrylamide gels, and used to immunize a rabbit. The specificity of the antibody obtained was confirmed by immunofluorescence and immunoblot analysis of a two-dimensional isoelectric focussing – SDS polyacrylamide gel as described earlier [18]. For the developmental analysis, samples of purified nuclear envelopes from different stages of liver development were separated on an SDS-polyacrylamide gel and then electroblotted onto nitrocellulose membranes [19]. The blots were incubated in blocking buffer (0.5% bovine serum albumin (BSA)/0.25% gelatin in phosphate-buffered saline (PBS)) for 2 h, followed by first antibody in 0.25% gelatin/PBS for 3 h. After several washes in 150 mM NaCl/0.1% NP-40/0.25% gelatin in PBS, the blots were incubated with alka-

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line phosphatase-conjugated anti-rabbit IgG at a dilution of 1:1000 for 2 h. After further washes, the blots were stained with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

2.5. RNA isolation and Northern hybridization

Total RNA was isolated from rat liver at different stages of development according to Chomczynski and Sacchi [20]. RNA samples were separated on a denaturing formaldehyde-agarose gel. Northern hybridization was carried out by standard procedures [21], using ^{32}P-labeled probes. Equal loading of RNA samples was initially assessed by staining of the agarose gel by ethidium bromide, and subsequently confirmed by hybridization with an actin probe.

2.6. Run-on transcription

Nuclei were isolated from adult, newborn and 15-day fetal liver [14] and used in run-on transcription assays as described [22], using α-[^{32}P]UTP to label the RNAs. The labeled RNAs were hybridized to 10 μg of linearized plasmid DNAs on dot blots and processed using standard procedures [21].

2.7. DNase I digestion of nuclei and Southern hybridization

Digestion of nuclei with DNase I under conditions wherein a general sensitivity to the nuclease could be analyzed was performed as described [23] with slight modifications. The nuclei were suspended in buffer containing 60 mM KCl/15 mM NaCl/0.05 mM $CaCl_2$/5 mM $MgCl_2$/0.1 mM EDTA/15 mM Tris-HCl, pH 7.4/0.5 mM dithiothreitol/0.25 M sucrose. A stock solution of DNase I was diluted in the following buffer from which $MgCl_2$ had been omitted: 60 mM KCl/15 mM NaCl/0.1 mM $CaCl_2$/15 mM Tris-HCl, pH 7.4/0.5 mM dithiothreitol. Different amounts of DNase I (0–30 Kunitz units/mg DNA) in 25 μl of DNase I dilution buffer were added to 475 μl of nuclei (20 A_{260}/ml). After incubation at 25°C for 5 min, the digestion was stopped by adding EDTA to a final concentration of 20 mM and chilling the tubes on ice. The acid solubility (in 0.9 M perchloric acid, 1.8 M NaCl) of the nuclei digested with 10–30 U DNase I was maintained in the range of 5–12%. DNA was isolated from nuclei after DNase I digestion as described [23] and analysed by agarose gel electrophoresis. Samples in which the extent of DNase I digestion was similar for fetal, new born and adult liver nuclei were further processed for Southern hybridization. The DNA was digested to completion with *Bam*HI, separated on a 0.7% agarose gel and subjected to Southern hybridization by standard protocols [21]. *Bam*HI was chosen as most of the genomic sequence of the lamin A gene appeared to be contained within two large *Bam*HI segments of 12 kb (intron 1 to exon 11) and 6 kb (exon 11, 12 and 3′ flanking sequences) (Q.A. Hamid, unpublished work).

Quantitative analysis of autoradiograms and blots was carried out on a Molecular Dynamics Computing Densitometer 300 A.

3. Results

3.1. Transcription of lamin A and C mRNAs

Total cellular RNA was prepared from different stages of developing rat liver as well as adult rat liver, and two cell lines, PCC-4 mouse embryonal carcinoma cells and F-111 rat fibroblasts. Although the embryonic liver had formed by day 13 after gestation, the earliest stage at which sufficient liver tissue could be obtained was at day 15. The RNA was analysed by Northern hybridization to several probes (Fig. 1A) and a quantitative analysis of the developmental profiles is shown in Fig. 1B. The prlamA probe hybridized to both lamin A (3.0 kb) and lamin C (2.5 kb) RNA. Significant levels of lamin A and C mRNAs in equivalent amounts were found in the 15-day fetus and these were maintained until day 18–19. At birth (day 20 or 21), there was a 10-fold decrease in both lamin A and C mRNAs. Throughout adult life these RNAs were expressed at very low levels. There was no detectable expression of lamin A and C transcripts in the embryonal carcinoma cell line, PCC-4, which is comprised of cells at the developmental stage of the inner cell mass in the early

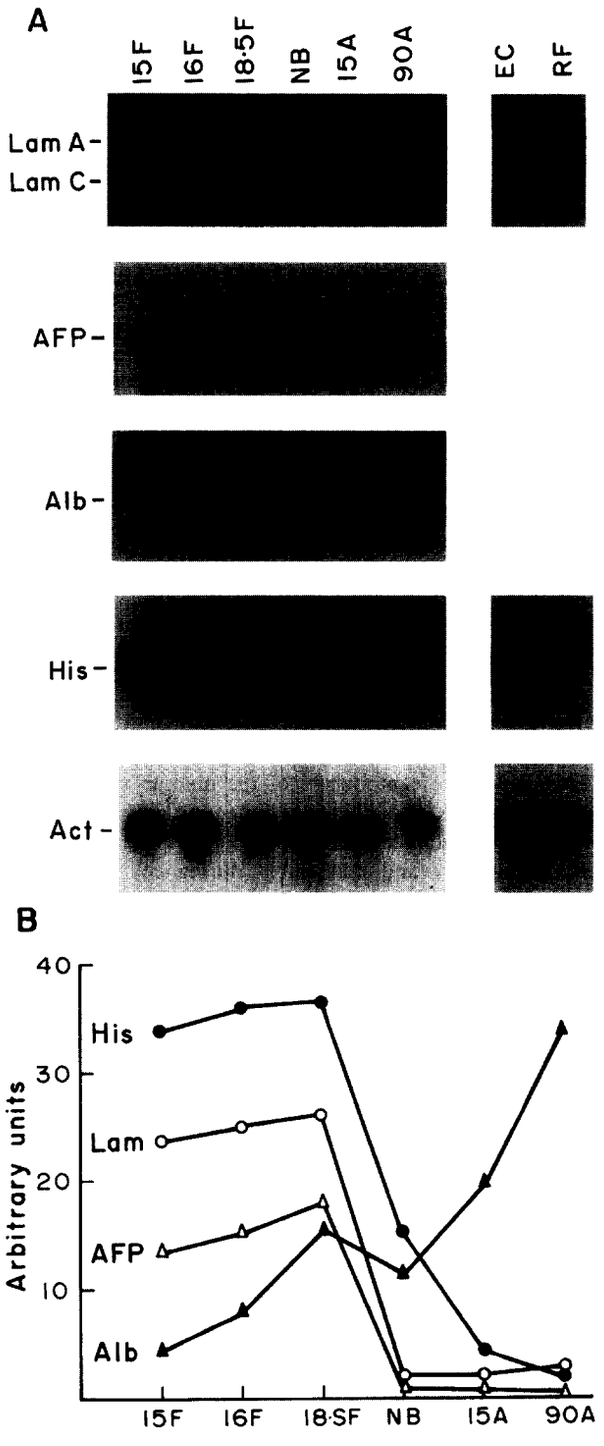

Fig. 1. Northern analysis of transcripts during liver development. (A) Total RNA (10 μg each) was isolated from fetal (15-, 16- and 18/19-day), newborn and adult liver (15- and 90-day), PCC-4 embryonal carcinoma (EC) and F-111 rat fibroblast (RF) cells, and subjected to Northern hybridization with probes for lamin A and C (lam A,C), α-fetoprotein (AFP), albumin (Alb), histone H4 (His) and β-actin (Act) mRNAs. (B) The autoradiograms of the developmental profiles were scanned on a densitometer; the results have been normalized with respect to the β-actin signals and the lamin A and C intensities have been combined for clarity.

blastocyst [24], and this is consistent with earlier immunocytochemical studies [5,6]. Considerable expression of lamin A and C mRNAs was evident in the rat fibroblast cell line F-

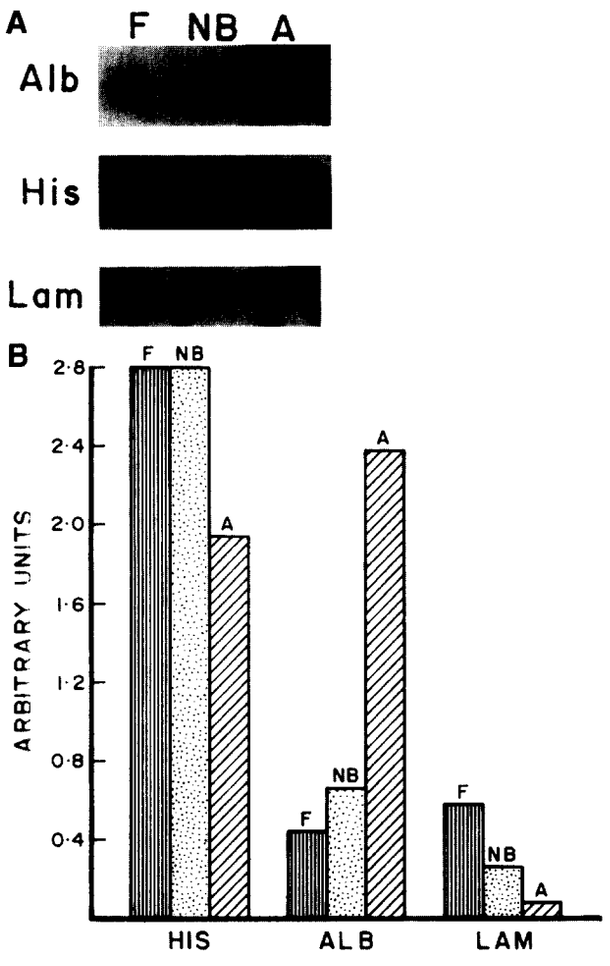

Fig. 2. Run-on transcription analysis. Labeled RNA was isolated from fetal (F), newborn (NB) and adult (A) liver nuclei and used to probe dot blots containing 10 μg per dot of pmalb2 (Alb), pHh4A-SP3 (His) or prlamA (Lam). The autoradiogram was scanned on a laser densitometer and the results (after correcting for background noise) are presented as a histogram.

111. In this cell line, the ratio of lamin A to C RNA has been occasionally found to be greater than one, but the reason for this anomaly is not known.

The developmental profiles of albumin and α-fetoprotein expression that were obtained in Fig. 1 are consistent with earlier reports [13,25]. The levels of α-fetoprotein mRNA (2.2 kb) were high in the fetus and reduced dramatically after birth, being undetectable in the adult animal. Although albumin mRNA (1.8 kb) was expressed to a lesser extent than α-fetoprotein in the early fetal stages, it gradually reached very high levels in the adult. (The albumin hybridization signal in the NB sample was unexpectedly 0.8-fold of what it should be.) Histone H4 RNA (0.8 kb) levels, which are a measure of cell division, fell rapidly after birth, coincident with a marked reduction in hepatocyte cell division. Both cell lines, PCC-4 and F-111, expressed considerable amounts of histone mRNA as expected, with higher levels being observed in the more rapidly dividing PCC-4 cells. The levels of β-actin mRNA (2 kb) were found to be in a similar range for all the samples, with slightly greater amounts in the first two lanes.

The pattern of lamin A RNA expression could be controlled at the level of mRNA transcription or its processing and degradation. In order to assess the role of mRNA transcription in the developmental appearance of lamin A RNA, run-on transcription assays were carried out with 15-day fetal, newborn and adult liver nuclei. The labeled RNAs were hybridized to plasmid DNA probes for the lamin A, albumin and histone genes. The results are shown in Fig. 2. Run-on transcript levels of lamin A are highest in fetal nuclei and decrease as development proceeds. This broadly correlates with the profile of steady-state lamin A RNA during development, although the decrease in steady-state RNA levels at birth is more substantial. The increase in albumin run-on transcripts correlates with the increase in mRNA steady-state levels during development. This is consistent with the albumin gene being under transcriptional control, as reported earlier [25]. On the other hand, histone H4 run-on transcript levels decrease only slightly in the adult, although steady-state histone H4 mRNA is barely detectable in the adult (as shown in Fig. 1). This can be attributed to the rapid degradation of most histone mRNAs after the cessation of DNA synthesis [26].

3.2. Expression of A-type lamin proteins during development

The detection of substantial amounts of lamin A and C RNAs early in fetal liver development prompted us to reexamine the expression of lamin proteins in developing liver by immunoblot analysis with anti-lamin A antibody. Previous immunocytochemical studies with whole tissue sections suggested that the A-type lamins were detectable only after birth in mouse liver [6]. We obtained a polyclonal antibody to bacterially expressed rat lamin A that predominantly recognized lamin A but also detected lamins B and C due to their high degree of homology. This polyclonal antibody was used to probe a blot of nuclear envelope proteins obtained from developing liver at different stages (Fig. 3). A duplicate blot was probed with a high affinity anti-lamin B antibody (described in [18]). These nuclear envelope preparations are enriched in lamins [15,16] (as shown in the Coomassie blue-stained gel in Fig. 3) and would allow their detection at a greater level of sensitivity. It was observed that the fetal liver nuclear envelopes were salt-extracted to a lesser extent than the adult liver nuclear envelopes during their preparation, and hence contained more intranuclear proteins. Equal numbers of envelopes were loaded per sample to offset this difference. A quantitative analysis of the immunoblot data is shown in Fig. 3B. Lamin B was expressed at high levels throughout development. The A-type lamins were detectable by day 15 of fetal liver development, continued to be expressed throughout fetal development and reached 2-fold higher levels in the 90-day adult. This is considerably earlier than that detected previously [6,18].

3.3. Chromatin structure of the lamin A gene

The degree of unfolding of the higher order structure of a gene, which is a measure of its transcriptional activity or potential to transcribe, can be measured by the extent of its digestion by a nuclease such as DNase I [27], under well-defined conditions of progressive digestion of chromatin [28]. In experiments designed to check the general DNase I sensitivity of the lamin A gene in liver tissue at different stages of development, nuclei were isolated from 15-day, newborn and adult rat liver and digested with 0–30 U/mg DNA of DNase I as described under Section 2. The time of digestion was main-

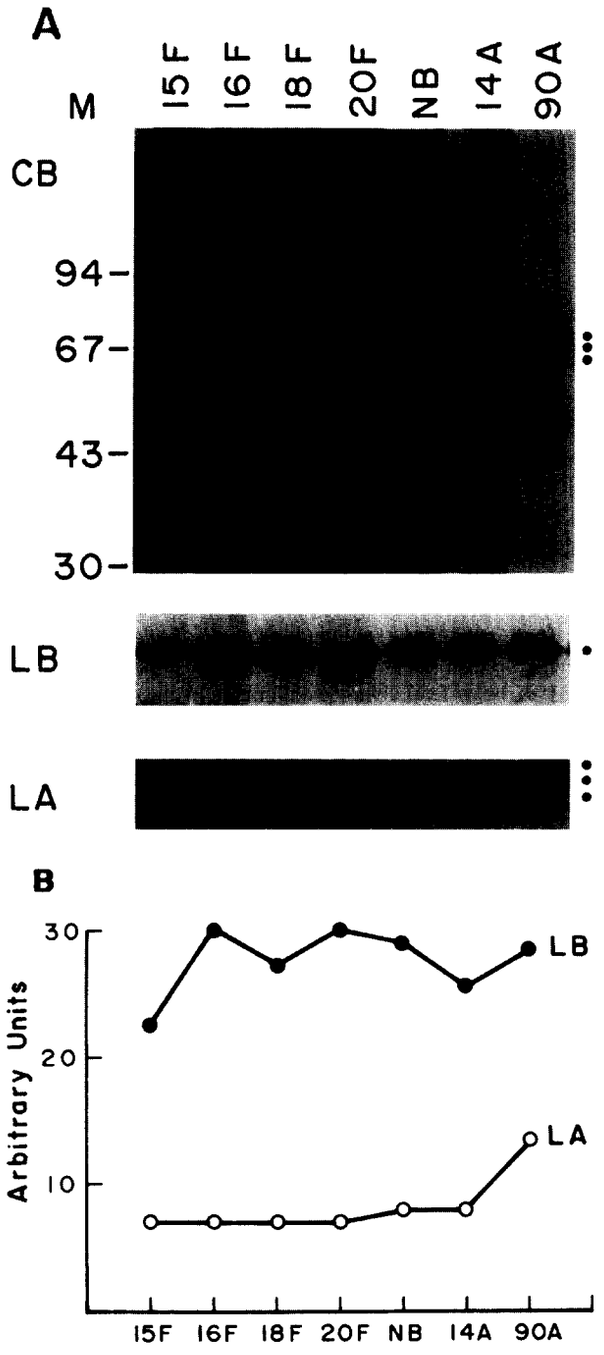

Fig. 3. Expression of lamin proteins during liver development. (A) Nuclear envelopes were isolated from fetal (15-, 16-, 18- and 20-day), newborn and adult liver nuclei (14- and 90-day); separated on a 10% SDS-polyacrylamide gel ($\sim 5 \times 10^6$ envelopes per lane) and either stained with Coomassie Blue (CB) or transferred to nitrocellulose membranes and probed with anti-lamin B antibody (LB), or anti-lamin A antibody (LA). Molecular mass markers (M): phosphorylase *b*, 94 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; and carbonic anhydrase, 30 kDa. Positions of lamins A–C in order of decreasing mass are indicated by dots. (B) The blots were scanned on a densitometer and results for LB and LA (only A-type lamins) are presented.

tained constant and the amount of DNase I increased so as to keep any contribution from endogenous nucleases at a uniform level [28]. It was confirmed that the DNA from the three stages of liver development was digested to a similar extent by DNase I (data not shown). The nuclease-digested DNA was further digested with *Bam*HI and subjected to Southern hybridization with probes for lamin A, albumin and α-fetoprotein in triplicate blots (see Fig. 4). A reduction in band intensity with increasing DNase I concentration indicates DNase sensitivity. The data has been quantitated as a percentage of the signal in the absence of DNase I for each sample. The analysis with the albumin and α-fetoprotein genes was carried out primarily to validate the methodology for the nuclease digestion. The 15 kb band encompassing the albumin gene is highly sensitive to DNase I in the adult where it is maximally transcribed and only partially sensitive in the newborn and insensitive in the 15-day fetus where it is transcribed at much lower levels. The α-fetoprotein gene (16 kb band) is nuclease-sensitive in fetal liver, partially sensitive in the newborn but nearly insensitive in the adult, consistent with its pattern of expression of RNA. The lamin A gene is highly sensitive to DNase I digestion (100% at 30 U DNase I) in fetal liver nuclei where it is maximally transcribed, remains 50% sensitive in the newborn as its transcription rate has reduced by this stage, but is only 20% sensitive in the adult wherein it is transcribed at low levels.

4. Discussion

In this report, we have described new findings on the activation of the A-type lamin gene during rat liver development. High steady-state levels of lamin A and C transcripts were detected by day 15 after gestation. Significant expression continued till birth, after which levels were reduced considerably. Analysis of run-on transcripts also indicated that the lamin A gene was transcribed in the 15-day fetus. Using polyclonal antibodies to the lamin proteins, both A-type and B-type lamin expression was detectable throughout embryonic liver development, as well as in the adult. Analysis of the chromatin structure of the lamin A gene revealed that it was highly nuclease sensitive at stages when the gene was maximally transcribed. These data suggest that the lamin A gene is activated early in fetal liver development, but its activity declines considerably in the adult.

Lamina-chromatin interactions have been proposed to have functional significance for the spatial organization of chromosomes during growth and development [1–3]. Studies carried out in several groups have established that the A-type lamin proteins are expressed only in differentiated cells [5–8]. Hence, the change in composition of the lamina from a predominantly B-type polymer to a heteropolymer of A and B-type lamins during cell differentiation may have important implications. Since the embryonic liver starts forming by day 11 of gestation in the mouse and rat, it was of some concern to us that lamin A and C proteins were detectable only after birth in mouse liver in an earlier report [6]. This observation might be attributable to the less sensitive technique of immunocytochemical analysis used in the study. To increase the sensitivity of our detection system we probed nuclear envelope preparations that are enriched in lamins. The A-type lamins could be detected at the earliest stage in rat liver development that was amenable to biochemical analysis, i.e. day 15 after gestation. Lamin B was expressed at high levels throughout development, as expected. This most probably corresponds to lamin B1, as this was the predominant sub-type detected in rat hepatocytes in an earlier report [29]. Northern analysis of the

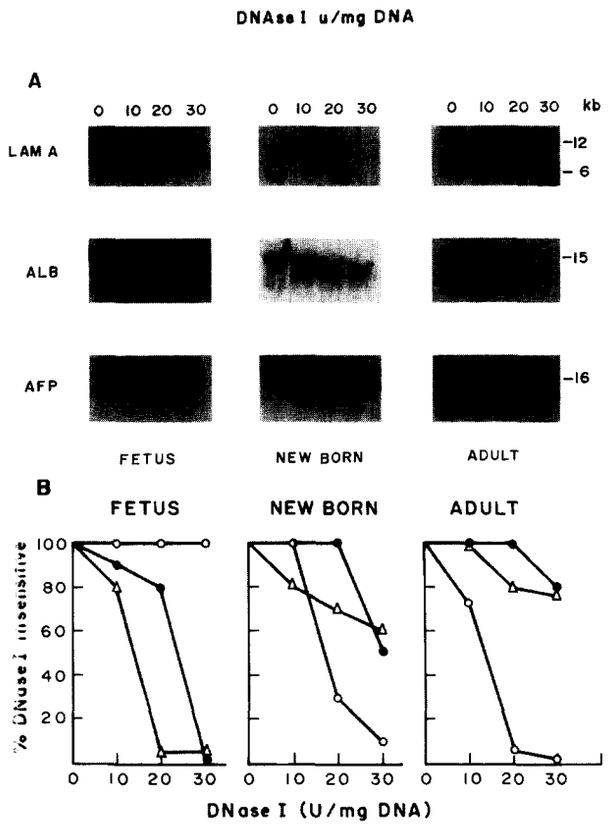

Fig. 4. DNase I sensitivity assay. (A) Nuclei from 15-day fetal, newborn and adult liver were digested with 0–30 U/mg DNA of DNase I, DNA was isolated and analyzed by Southern hybridization in triplicate blots, using probes for lamin A (lam A), albumin (ALB) and α-fetoprotein genes (AFP). (B) The autoradiograms were scanned on a densitometer and the results plotted as a percentage of the signal in the absence of DNase I for each sample. The signals for the two bands for the lamin A gene have been combined in the analysis. (●) lamin A; (○) albumin; (△) α-fetoprotein.

lamin A gene demonstrated that it was abundantly expressed during embryonic development of liver, when the fetal liver cells are proliferating rapidly. As the liver cells cease division in the adult (indicated by a decrease in histone H4 mRNA levels), lamin A RNA levels also decline substantially. Minimal amounts of lamin A RNA appear to be sufficient to maintain the adult lamina, indicating that the lamins have a slow turnover in the adult.

In studies on the expression of lamins A and C in P19 embryonal carcinoma cells induced to differentiate with retinoic acid, Lanoix et al. [30] reported low levels of lamin A and C RNAs in uninduced cells, which increased several-fold upon induction of differentiation with retinoic acid, but observed no change in run-on transcript levels. The authors proposed a post-transcriptional control of A-type lamin expression based on these results. However, Mattia et al. [31] failed to detect any lamin A and C RNA in uninduced P19 and F9 embryonal carcinoma cells in their studies, whereas these mRNAs were induced upon treatment with retinoic acid; hence, the authors suggested that A-type lamin expression is regulated at the transcriptional level. They argue that the presence of low levels of lamin A and C RNAs in embryonal carcinoma cell lines could be attributed to a small population of spontaneously differentiated cells that arise occasionally in these lines. We have also not detected any lamin A and C RNA in PCC-4 embryonal carcinoma cells. In a recent study [12], it has been proposed that the expression of *Drosophila* lamin C (the only A-type lamin identified in this species) is regulated at the transcriptional level during development.

The albumin and α-fetoprotein genes have served as excellent models for studies on liver-specific and developmentally regulated gene expression [13,25], especially at the transcriptional level. Fetal hepatocytes (day 15–17) express high levels of α-fetoprotein, produce albumin but do not express tyrosine aminotransferase which is characteristic of mature adult hepatocytes. Our observations on the expression of lamin A in the 15-day fetal liver thus suggest that activation of the lamin A gene is an early event in the maturation of the hepatocyte. By in situ hybridization studies, expression of the α-fetoprotein gene has been detected in hepatoblasts at day 11.5, prior to completion of liver morphogenesis [32]. The earliest stage in liver differentiation at which lamin A expression can be detected has not yet been ascertained. Although 20–30% of embryonic liver cells are hematopoietic cells and the remainder are fetal hepatocytes, the expression of lamin A would be derived primarily from the hepatocytes since immature blood cells do not express A-type lamins [33,34].

The transcriptional activation of a gene would require the initial decondensation of a domain of chromatin containing the gene This unfolding of the higher order structure can be conveniently studied by the use of endonucleases such as DNase I [27,28,35]. The correlation of tissue-specific differences in general DNase I sensitivity with gene transcription has been well-documented for several genes such as globin, ovalbumin, albumin and α-fetoprotein [13,27]. In addition, enhanced DNase I sensitivity or hypersensitivity is observed in the 5′ promoter region of active genes [35,36]. Since several factors are likely to contribute to the DNase sensitivity of chromatin, such as altered conformation of nucleosomes, binding of specific proteins or changes in DNA conformation, the reduction of band intensity as a function of DNase I concentration may not be exactly comparable for different genes. Hence, it is more appropriate to compare the trend of sensitivity in different tissues or developmental stages for a particular gene. We have compared the general DNase I sensitivity of the α-fetoprotein, albumin and lamin A genes at different stages of liver development, using probes that hybridize to large genomic segments encompassing most of the coding sequences of these genes. Our data on the patterns of DNase sensitivity of the α-fetoprotein and albumin genes correlate well with their transcriptional status during liver development and are consistent with earlier studies [13]. The results on the DNase sensitivity of the lamin A gene suggest that the gene is in an active chromatin conformation by day 15 of gestation when it is maximally transcribed, and is partially unfolded in the newborn as its transcription rate has reduced by this stage. The lamin A gene adopts a largely inactive conformation in the adult wherein it is transcribed at very low levels.

In conclusion, we have established that the lamin A gene is expressed early in rat liver development and is in an open, active chromatin conformation at this stage. We have also been able to detect lamin A protein by day 15 after gestation by Western blot analysis of nuclear envelopes obtained from liver nuclei. Our results suggest that the mechanisms contributing to lamin A regulation operate primarily at the transcrip-

tional level in the fetus. Further studies are in progress to characterize the promoter sequences of the lamin A gene.

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