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Short Contribution

Characterisation of developmentally regulated chromatin structure in the coding region of the proto-oncogene, c-*fos*, in the male laboratory mouse

CHANDRASEKHAR KANDURI* and RAJIVA RAMAN

Cytogenetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi, India

ABSTRACT In mouse, tissue-specific developmental *de novo* methylation of the proto-oncogene c-*fos*, which is abundantly expressed during embryonic stages, occurs perinatally (between the day of birth to 20 dpp) and is maintained in the adult. In liver, where c-*fos* is only active up to the day of birth, the gene has more sites methylated than in brain, where it is expressed until about day 5 post-partum. We have studied chromatin organisation of c-*fos* and compared this to DNA methylation in the fetal and adult brain and liver. Purified nuclei of these tissues from fetus as well as adult were digested with the restriction enzyme *Msp*I. DNA was extracted from the *Msp*I digested chromatin and probed with two DNA segments covering the major part of the body of the gene (from distal part of second exon to major part of fourth exon). Southern hybridisation studies revealed that in the fetus, in both liver and brain, the chromatin in the coding region was sensitive to *Msp*I digestion and the extent of sensitivity was nearly the same between the two. In the adult tissues, however, chromatin from brain was almost as sensitive as in the fetus, but in the liver it was highly resistant to *Msp*I. We suggest that a shift from the undermethylated state in the fetus to the heavy methylated state in the adult causes a corresponding change in the organisation of chromatin of c-*fos* in the coding region. Furthermore, the difference in the tissue-specificity in the methylation induced chromatin compaction could be due to differences in the transcription levels of c-*fos* and *de novo* methylation during early neonatal development.

KEY WORDS: *DNA methylation, chromatin, c-fos*

Methylation of cytosine residues in the dinucleotide CpG is the most prevalent epigenetic modification of the mammalian genome. Over the last two decades, a considerable amount of data has accumulated to demonstrate the role of DNA methylation in gene regulation, chromatin organisation and genomic imprinting (Keshet *et al.*, 1986; Reik *et al.*, 1987; Sapienza *et al.,* 1987; Cedar, 1988). Such studies have suggested that the chromatin harbouring methylated DNA has a strikingly different conformation from that having unmethylated DNA and this parallels the changes associated with active and inactive chromatin (Ball *et al.*, 1983). The crucial evidence in this regard comes from the *in vitro* gene transfer experiments of Buschhausen *et al.* (1987). Here, methylated Hsvtk gene constructs introduced into Ltk-cells, remain active until about 48 h after the injection after which they become repressed. In contrast, the unmethylated constructs do not show any repression. They concluded that a specific chromatin structure is necessary for the methylated DNA to become effective in gene regulation. Later studies (Kass *et al.*, 1997) have shown that the transcriptional repression of methylated templates occurs during transcription initiation, involving time-dependent assembly of nucleoprotein complexes which are able to inhibit transcription more efficiently than nucleosome alone. The recent demonstration of association of the methyl CpG-specific protein (MeCP2) with histone deacetylase (Jones *et al.*, 1998; Nan *et al.,* 1998) partly clarifies the role of DNA methylation in chromatin organisation.

In a recent study on the dynamics of *de novo* methylation of individual cytosines during development, we have shown that in the coding region of the proto-oncogene, c-*fos*, most of the *Hpa*II and *Hha*I CpG sites (*viz*., m3, m4, m5, m6 and m7; see physical map in Fig. 1) are undermethylated in fetal somatic tissues such as brain, kidney, liver and spleen, but are heavily methylated in adults in a tissue-specific manner. Interestingly, this methylation occurs stepwise and unidirectionally, between day 18.5 (dpc) of fetal development to day 20 after birth (Chandrasekhar and Raman, 1997) and once established, this pattern is maintained through adult life. In view of the correlation between the status of DNA methylation and chromatin organisation, it was of interest to know whether the shift

Abbreviations used in this paper: Dnmt-1, DNA methyltransferase-1; dpc, days post-coitum; dpp, days post-partum.

***Present and corresponding address for reprints:** Department of Animal Development and Genetics, Uppsala Universitet, Norbyvägen 18A, S-75236, Uppsala, Sweden. FAX: 46 18 4712683. e-mail: kanduri.chandrasekhar@devbiol.uu.se

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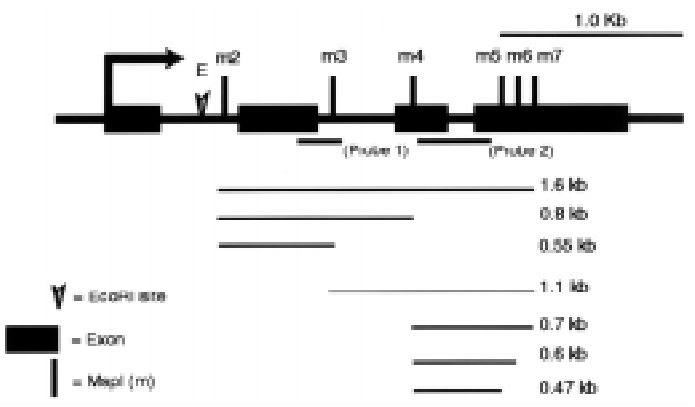

Fig. 1. Physical map of the *c-fos* gene showing positions of *Msp*I sites. *Black boxes represent exons. The lines drawn below the map denote the fragments resulting from the accessibility or inaccessibility of the MspI sites (m) at the chromatin level after hybridisation with probes 1 and 2.*

from the unmethylated state in the fetus to the methylated state in the adult correlated with a change in the organisation of chromatin of c-*fos*. We have performed an *Msp*I sensitivity assay on isolated nuclei of brain and liver from fetal and adult mice. The restriction enzyme, *Msp*I, cleaves naked DNA at the sequence 5'CCGG 3', regardless of whether the internal C is methylated or not. When DNA is organised into chromatin, however, it is resistant to *Msp*I if the internal C is methylated, possibly due to chromatin alteration brought about by the methylated CpG-binding protein(s) (Antequera *et al.,* 1989).

Figure 2B and C shows the *Hpa*II restriction pattern of DNA from fetal and adult tissues of brain and liver, as compared to the *Msp*I pattern in Figure 1A. In c-*fos*, the process of methylation in liver starts late in fetal life (after 18.5 dpc) and is completed neonatally by 20 dpp, involving sites m7 to m3. In brain, c-*fos* methylation starts only after birth, between day 5 and 20 post partum and only up to m4, leaving m3 unmethylated even in the adult. Methylation patterns vary between liver and brain, therefore, temporally, as well as qualitatively, both in fetus and in adult.

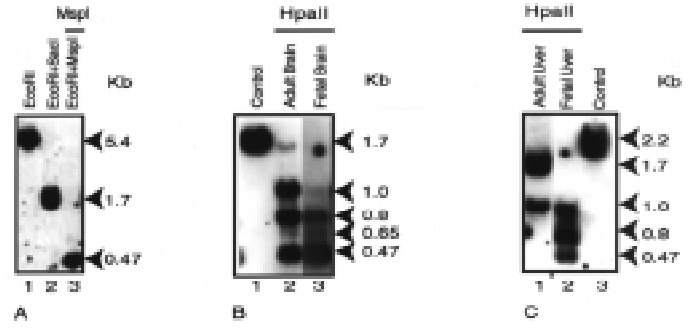

Fig. 2. Southern profiles of naked DNA digested with *Msp*I and *Hpa*II. **(A)** *Southern blot showing the MspI pattern of genomic DNA probed with probe 2. Lane 1 contains EcoRI digested DNA; lane 2, EcoRI/SacI digested DNA; lane 3, EcoRI/MspI digested DNA.* **(B)** *Southern blot showing the HpaII restriction pattern of fetal and adult brain DNA. Lane1, EcoRI/SacI digested DNA (Control); lane 2, HpaII digested adult brain DNA and lane 3 contains HpaII digested fetal brain DNA.* **(C)** *Southern blot showing the HpaII pattern of fetal and adult liver DNA. Lane 1 contains HpaII digested adult liver DNA; lane 2, HpaII digested fetal liver DNA and lane 3 HincII/SacI digested DNA (control).*

Isolated nuclei from fetal (16.5 dpc) and adult (above 70 day) brain and liver were digested with increasing concentrations of *Msp*I (50, 100 and 200U/ml) for 60 min at 37°C. After treatment, the DNA was extracted and digested with *Eco*RI to obtain a 5.4 kb fragment that spans the region from the distal end of intron 1 to about 2 kb downstream of the gene. The DNA was hybridised with an α-^{32}P-labelled 248bp *Pst*I fragment (probe 1; comprises distal part of exon 2 and part of intron 2) or a 365bp *Msp*I fragment (probe 2; parts of exon 3 and 4 and intron 3), mainly to detect the accessibility of *Msp*I sites in the coding region. The portion of the gene covered by *Eco*R1 digestion has six *Msp*I sites (Fig. 1). The lines drawn below the physical map in Figure 1 denote the fragments resulting from the accessibility or inaccessibility of the *Msp*I (m) sites at the chromatin level after hybridisation with probes 1 and 2. When DNA from adult liver was digested with 50U *Msp*I and probed with probe 2, it yielded only one prominent band of 4.8 kb (it is merged along with the 5.4 kb band) showing inaccessibility at most of the *Msp*I sites present in the coding region (Fig. 3, lane 3). At higher concentrations, however, an additional faint band appeared at 1.4 kb (Fig. 3, lanes 4-6). In

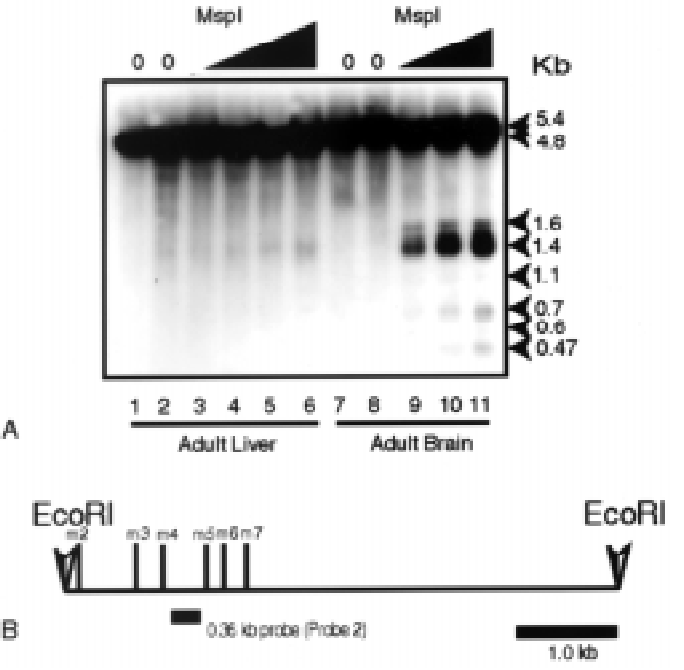

Fig. 3. Chromatin organisation in the 3'end of *c-fos* coding region in adult liver and brain analysed with Probe 2. (A) *Southern blot of MspI treated nuclear DNA from adult liver and adult brain probed with Probe 2 shows the absence of MspI sensitive chromatin in the adult liver. Lanes 1-6, contain DNA from nuclei treated with increasing concentrations of MspI (50,100, 200 U/ml) from adult liver and, adult brain (lanes 7-11).* **(B)** *Physical map showing the locations of MspI sites. The line drawn below the map is the probe used for hybridisation.*

contrast, multiple bands were obtained in the adult brain (4.8, 1.6, 1.4, 1.1, 0.7, 0.6 and 0.47 kb), even with 50U but more prominently with 100 and 200U of *Msp*I (Fig. 3, lanes 7-11). The *Msp*I-digested naked DNA is expected to yield a 0.47 kb fragment with probe 2 as shown in Figure 2A. When the chromatin was analysed with probe 1 (covering a region 5' to probe 2), a comparable picture emerged in both liver and brain (the smallest fragment in the *Msp*I treated brain nuclei was 0.55 kb) (Fig. 4, lanes 1-11). Thus, while most of the *Msp*I

sites of the c-*fos* coding region are accessible at the chromatin level in the adult brain (though to various degrees), they are inaccessible in liver, both within the 5´ and 3´ part of the coding region.

That this difference in the adult tissues is engendered simultaneously or subsequent to the methylation in fetal life is obvious from similar analysis of brain and liver from 16.5 dpc fetus, the stage at which the analysed sites were undermethylated in both tissues. Unlike in the adult, fetal liver generates a series of fragments (4.8, 1.6, 1.4, 1.1, 0.8, 0.7, 0.6 and 0.47 kb) with probe 2 (Fig. 5A, lanes 1-5). Fetal brain showed a similar pattern (Fig. 5B, lanes 1-5). With probe 1 multiple bands (4.8, 1.6, 1.4, 0.8 and 0.55 kb) were also obtained from brain and liver (Fig. 6A, lanes 1-5; Fig. 6B, lanes 1-5). While the brain chromatin gives prominent lower molecular length bands with 50U of *Msp*I, in liver they appear prominently with 200U of the enzyme indicating that *Msp*I sites in the 5' part of the coding region are more accessible to *Msp*I in the fetal brain nuclei than in fetal liver. In fetal tissues, therefore, where it is undermethylated, the c-*fos* chromatin is more accessible to *Msp*I than in the adult. The fact that *de novo* methylation starts earlier in liver than in brain may explain a relatively more compact chromatin in fetal liver. It is evident from the present results that the chromatin organisation of c-*fos* undergoes a developmental change, being more open in the fetus (when the gene is undermethylated and is expressed in various tissues) than in the adult. The chromatin organisation of c-*fos* coding region in the adult is of interest, however, since the gene is inactive in both tissues, it is only in liver that the chromatin is condensed enough to be inaccessible to *Msp*I; the brain is almost as sensitive to the enzyme as it is in the fetus where the gene is active and undermethylated. Although there is a strong correlation between the state of activity, DNA

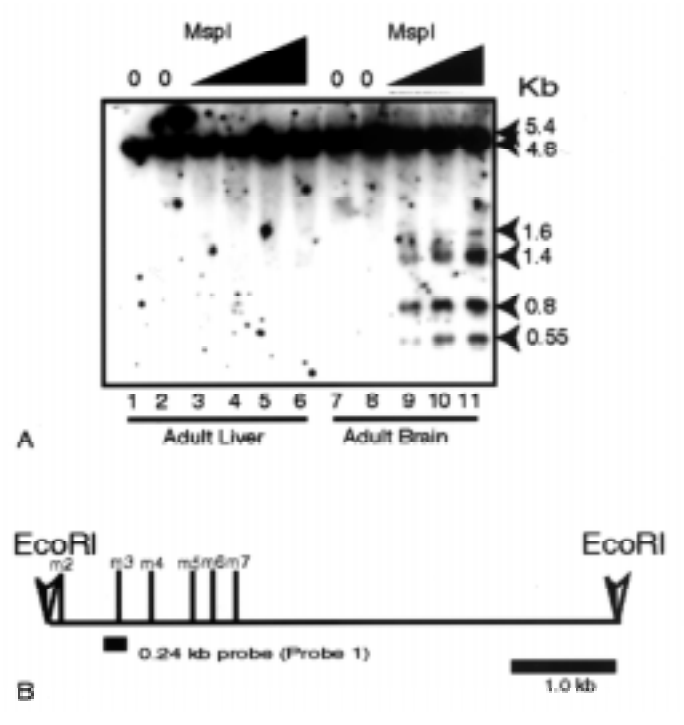

Fig. 4. Chromatin organisation in the 5′end of *c-fos* coding region in adult liver and brain analysed with Probe 1. (A) *Blot from Figure 3A rehybridised with Probe 1, also shows the absence of MspI sensitive chromatin in the 5′ part of the coding region.* **(B)** *Physical map showing the locations of MspI sites. The line drawn below the map is the probe used for hybridisation.*

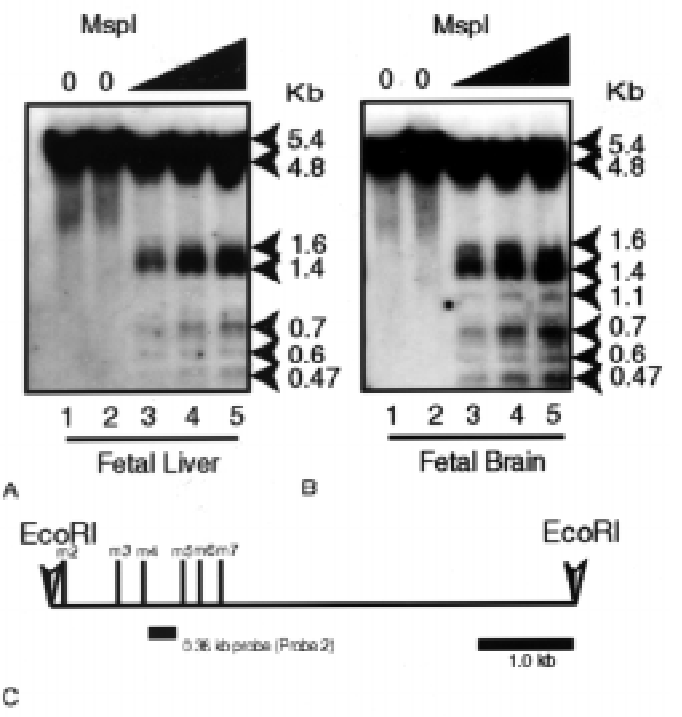

Fig. 5. Chromatin organisation in the 3′end of c-*fos* coding region in fetal liver and brain analysed with Probe 2. (A *and* **B)** *Southern hybridisation of MspI treated nuclear DNA from fetal liver (Fig. 5A, lanes 1-5), and fetal brain (Fig. 5B, lanes 1-5) with probe 2 shows the presence of MspI sensitive chromatin in both fetal liver as well as fetal brain.* **(C)** *Physical map showing the locations of MspI sites. The line drawn below the map denotes the probe used for hybridisation.*

methylation and chromatin compaction in c-*fos*, however, do not appear to be a direct or causal link. It is possible that the observed *de novo* methylation-induced chromatin compaction in the c-*fos* coding region could be a consequence of transcriptional repression. The latter contention was further corroborated in a recent study which analysed tissue-specific genes that were earlier shown to be regulated by DNA methylation in Dnmt-1 knockout mice. This revealed that the methylation patterns at the promoter regions of tissue-specific genes are a consequence of transcriptional inactivation rather than a cause (Walsh and Bestor, 1999). One major difference between the patterns of methylation in brain and liver, however, seems to be important in the reorganisation of chromatin. While in brain methylation spreads only up to m4, leaving m3 completely unmethylated, in liver it extends up to m3 site which is heavily methylated in the adult. As such, liver is the only somatic tissue in which m3 is methylated (Chandrasekhar and Raman, 1997). This is in agreement with the present report that the *Msp*I sites in the 3' and 5' part of the coding region of c-*fos* gene are equally inaccessible to the *Msp*I in adult liver while in brain the *Msp*I sites in the 5' part of the coding region are more readily accessible than in the 3' part both in the fetal and adult brain.

We assume that the tissue-specific difference in the methylation-induced chromatin compaction could be due to the difference in the c-*fos* transcription levels. Previous reports on c-*fos* gene expression show that it is transcribed up to day 18.5 dpc in all the analysed somatic tissues, such as liver, kidney and spleen, whereas its expression in the brain continues to rise until day 5, after which it decreases (Kasik *et al.*, 1987). In light of the fact that transcription

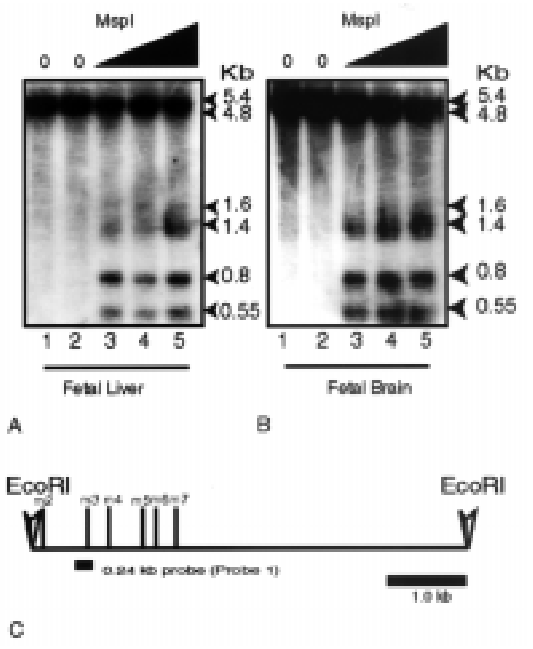

Fig. 6. Chromatin organisation in the 5'end of *c-fos* coding region in adult liver and brain analysed with Probe 1. (A *and* **B)** *Blots in Figure 5A and B were reprobed with Probe 1. They also show the presence of MspI sensitive chromatin in the 5' part of the coding region of c-fos gene.* **(C)** *Physical map showing the locations of MspI sites. The line drawn below the map is the probe used for hybridisation.*

factors such as SP1, NF-kB and GAL4-VP16 are capable of demethylating the DNA sequences adjacent to their recognition elements (Matsuo *et al.*, 1998), we believe, therefore, that the ongoing transcription in brain during the perinatal development, may have prevented *de novo* methylation. This generates undermethylation patterns and as a consequence, *Msp*I sensitive chromatin structure. This is further supported by our earlier observation of the under methylation of *Hha*I and *Msp*I sites flanking the SP1 motif. Therefore, the present study, using c-*fos* proto-oncogene as a model system, addresses the relation between chromatin organization and DNA methylation.

Experimental Procedures

Random bred mice of Parkes strain reared under constant dark and light cycles were used during the entire study. Liver and Brain from fetus (16.5 dpc) and adult (above 70 days old) were collected, taking the date of vaginal plug formation as day 0.5.

Isolation of nuclei and MspI treatment

Nuclei were isolated according to Antequera *et al.* (1989) with slight modifications. Liver and Brain were dissected out and homogenised with 30 strokes of a tight fitting pestle of the Dounse homogenizer in the homogenising buffer (10 mM Tris-HCl pH 7.5, 125 mM KCl, 25 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF, 1 mM DTT, 0.25 M Sucrose and 0.1% NP-40). The homogenate was filtered through 8 layers of cheese cloth and left on ice for 10 min. The homogenate was layered on a 1.5 mM sucrose cushion and was spun at 7500 rpm for 10 min at 4°C. The nuclei pellet was resuspended in the homogenising buffer without NP-40. Approximately $1x10^7$ nuclei were aliquoted into eppendorf tubes and washed twice with *Msp*I buffer (10 mM Tris-HCl pH7.5, 100 mM NaCl, 5 mM $MgCl_2$ 0.1 mM PMSF and 1 mM DTT). Finally, the nuclear pellet was resuspended in 300 µl of *Msp*I buffer. The nuclei were treated with 50U, 100U and 200U/ml of *Msp*I for 60 min at 37°C. After *Msp*I digestion, DNA was extracted using the phenol chloroform method.

Southern hybridisation

Fifteen to 20 µg of DNA were treated with 6U/µg of the enzyme, *Eco*RI, (New England Biolabs, USA) according to the manufacturer's recommendation. The digested DNA was electrophoresed in a 1% agarose gel in TBE buffer and transferred to a nylon membrane (S&S Nytran). The membrane was baked at 80°C for 2 h prior to hybridisation.

Plasmid probes (365bp *Msp*I and 248 *Pst*I fragments; see physical map in Figure 1) were labelled with (α-^{32}P) dCTP (Specific activity 3000 Ci/mM; BRIT, Bombay) using the Random Priming System (NEB-BLOT kit, New England Biolabs, USA) to a specific activity more than $1x10^8$ cpm/µg. Blots were hybridised using $2.5x10^7$ cpm for 100 cm^2 (probe concentration, 3-5 ng/ml of hybrid mix) for 16 to 20 h at 42°C. Hybridisation buffer comprised of; 50% formamide, 6xSSC, 5xDenharts, 0.5% SDS, 200 µg/ml Salmon sperm DNA. The hybridised blots were washed in 0.5xSSC, 0.1% SDS at 65°C, and autoradiographed.

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