ARTICULOS ORIGINALES COMPLETOS/ FULL ORIGINAL PAPERS

DNase I - FOOTPRINTING: A METHOD FOR THE CHARACTERIZATION OF POSSIBLE TRANSCRIPTIONAL SIGNALS IN AN EARLY REPLICATED HUMAN DNA SEQUENCE

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SUMMARY

DNaseI- footprinting is a method for the detection of protein-DNA binding specificity. The technique is a simple conjoining of the Maxam-Gilbert DNA-sequencing method and the technique of the DNase-protected fragment isolation. Fragments of 5' end-labeled, double-stranded DNA segment, partially degraded by DNase in the presence and absence of the binding protein, are visualized by electrophoresis and autoradiography alongside the base-specific reaction products of the Maxam-Gilbert sequencing method.

It is then possible to see the protective "footprinting" of the binding protein on the DNA sequence. The presence of binding sites for nuclear factors related to transcription signals were detected by the footprinting method in a transcribed genomic region, replicated at the onset of S- phase in human HL-60 cells.

RESUMEN

El método de "footprinting" es usado para la detección de uniones específicas ADN-proteína. Esta técnica consiste en la unión del método de secuenciación de Maxam-Gilbert y la técnica de aislamiento de fragmentos protegidos a la acción de la ADNasa. Fragmentos de ADN de doble cadena marcados radioactivamente en su extremidad 5', parcialmente degradados por la ADNasa en presencia y ausencia de un extracto nuclear son separados por electroforesis. Mediante el uso de la técnica de secuenciación de Maxam-Gilbert es posible la localización, a nivel de base-específica, de zonas protegidas a la acción de la ADNasa por la unión de la proteína en cuestión a la secuencia de ADN particular.

La presencia de sitios de reconocimiento en el ADN para factores nucleares relacionados con señales transcripcionales fue detectada por el método de "footprinting" en una región de ADN humano, transcripta activamente y la cual es replicada al inicio de la fase S del ciclo celular en células HL-60.

INTRODUCTION

The specificity of DNA-protein interaction, as a fundamental recognition process in molecular biology, has been the aim of several experimental methods designed to characterize this specificity (Gilbert *et al.*, 1971; Gilbert, 1976). The footprinting method is a simple and specific technique for DNA-binding protein detection and determination of the specific base for this protection.

The footprinting technique, supports the isolation and characterization of a fragment of DNA which is protected from DNase degradation by the DNA-binding protein (David and Schmitz, 1978). In this article we report human sequences which have binding sites for nuclear factors present in nuclear extract of HL-60 cells. One of this sequences shows a remarkable homology with an upstream element in the Major Late promoter of Adenovirus 2, which is recognized by a protein USF-MLTF (Carthew *et al.*, 1985; Sawadogo and Roeder, 1985) which acts as an upstream binding element in the transcription activation.

Our finding supports previous experiments in which a human genome region, replicated at the onset of S-phase was isolate, and a DNA fragment capable of promoting transcription (Falaschi and Biamonti, 1988) has been located in this region.

MATERIALS AND METHODS

Cells and cell nuclear extract preparation

HL-60 human promyelocytic cells were cultured in RPMI 1640 (GIBCO) supplemented with 10% fetal calf serum and 2 mM

L-glutamine. Preparation of nuclear extract was performed by the method of Dignam (Dignam *et al.*, 1983).

DNA sequencing and labelling of DNA with ^{32}P

Single subclones were end-labeled and sequenced essentially as described by Maxam and Gilbert (Gilbert *et al.*, 1971).

DNase footprinting assays

DNase footprinting assays were performed essentially as described by (Jones *et al.*, 1985).

Fragments of 100-300 bp used in this study were prepared using different utility restriction sites from pB48, a clone isolated from a genomic library of human placenta DNA (Tribioli *et al.* , 1987) and analyzed by the footprinting method for both DNA strands (figure 1).The fragments were treated with alkaline phosphatase (Boehringer) according to (Sambrook *et al.*, 1989) followed by end labelling with ^{32}P and were eluted from polyacrylamide gels (Jones *et al.*, 1985).

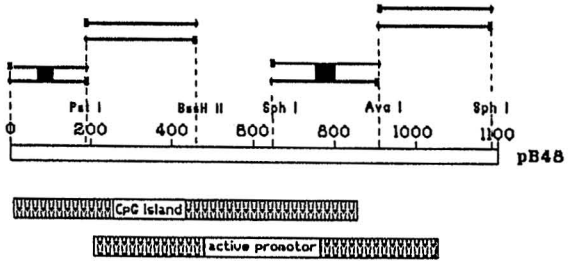

Fig.1 Map of pB48 human DNA insert and footprinting strategy. pB48 is a clone (1100 bp) isolated from a library of human placenta DNA. Dots indicate sites of end-labelling; arrows indicate the direction of footprinting assay; boxes shade shows the protected regions. The CpG island and the active promoter on the insert are showed in figure.

Binding reaction

One ng (10^4 cpm) of end-labeled restriction fragments were incubated with increasing quantities of HL-60 nuclear extract at a final protein concentration of 25, 50 and 100 μg, and 3 μg of Poly[d(I-C)]:Poly[d(I-C)] (Boehringer) in 10 mM Tris-HCL pH 7.5; 50 mM NaCl; 5 mM $MgCL_2$; 1 mM EDTA; 1 mM DTT; 0,2% Triton X-100; 5% Glycerol (final volume 50 μL).

After 30 min incubation at room temperature 50 μL of 5 mM $CaCl_2$ was added. A reaction without nuclear extract was used as negative control.

DNase digestion

Freshly diluted DNase I was added at a final concentration of 0.01-0.025 μg/mL in the negative control. The DNase I-nuclear extract ratios used in the different points were:

DNase I (final conc.)	:	Nuclear Extract
1 μg / mL		25 μg
2 μg / mL		50 μg
4 μg / mL		100 μg

The digestion was allowed to proceed 1 min at room temperature and then, 100 μL of stop solution were added (0,6 M NaCl; 0,1 mg/mL Salmon sperm DNA; 0,2 % SDS; 10 mM EDTA pH 8). Phenol Chloroform extraction was carried out and the products of the reaction were analyzed as previously described.

RESULTS

In view of the indications that pB48 insert contains different regulatory signals, an active promoter overlapping a putative CpG island (Falaschi *et al.*, 1988) and that it derives from an expressed region of the human genome (Biamonti *et al.*, 1991), we searched binding sites for specific nuclear factors mapping a region of 1100 bp which encompasses the active promoter and the CpG island region by the DNaseI-footprinting assay. Different fragments were analyzed and the results show two protected regions at the Sph I- Ava I (780 nt-801 nt) and Eco RI- Pst I (138 nt-163 nt) fragments (figure 1). These protected regions are visible in both DNA strands.

The sequence 5'TTCGTCACAGTGATGCGACCG 3' shows a remarkable homology (except for one mismatch) with an upstream element in the Major Late promoter of Adenovirus type 2 (Carthew *et al.*, 1985).

The protected sequence is show in figure 2 A. Interestingly another protected sequence 5'CACTTCCGGAAACACTTGTCCGTCAT 3' is palindromic and presents in the central part, at the GA di-nucleotide, an hypersensitive site for the DNase I (figure 2B). This sequence was analyzed using the data bank in order to detect homology with sequences related to transcriptional signals. But our efforts were null in this sense. However, the feature of enclosing a hypersensitive site for DNase-I flanked by palindromic sequences, summed to the fact that this site is located in a region highly transcribed (Biamonti *et al.*, 1991), make it a strong candidate to play a role as a regulation element (positive or negative) in the transcriptional process because hypersensitive sites are noted over DNA immediately upstream of the transcription initiation site and these reflect the binding of the various proteins required to establish and maintain transcriptional competence (Elgin, 1988; Assendelft *et al.*, 1989).

Furthermore, the sensitivity to DNase is related to specific regions of chromatin which take an open conformation as a cause or consequence of the binding of determined nuclear factors (Kageyama and Pastan, 1989; Suaren and Chalkley, 1990; Englander and Wilson, 1990). Concerning to location of two

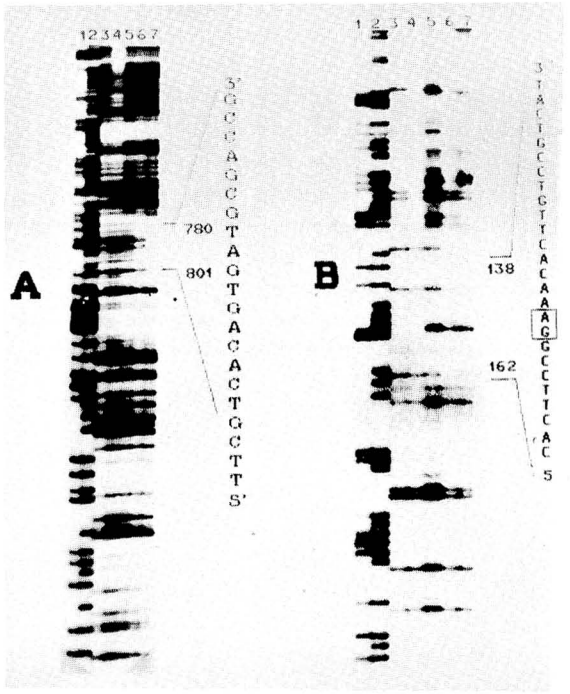

Fig.2 Characterization of DNA binding sites for nuclear factors at a human genomic region. (A) DNase I-footprinting analyses the 195 bp Sph I-Ava I fragment of pB48. The fragment was end-labeled, incubated with increasing quantities of HL-60 nuclear extract, digested with DNase I and loaded onto a sequencing gel. The Maxam-Gilbert sequence and all the procedure were carried out as described in Materials and Methods. Lane 1-2: G and G+ A Maxam-Gilbert sequence. Lane 3-4: Shows the control DNase digestion pattern with no protein factors added. Lane 5-7 : Shows the binding reaction with 25 μg, 50 μg and 100 μg of nuclear extract respectively. On the right protected oligonucleotide sequence is indicated. (B) The 250 bp Eco RI- Pst I fragment of pB48 was subjected to DNase I- footprinting assays as described above. Lane 1-2: G and G+ A sequence. Lane 3-4: Shows the control DNase digestion pattern with no protein factors added. Lane 5-7 : Shows the binding reaction with 25 μg, 50 μg and 100 μg of nuclear extract respectively. The nucleotide sequence protected is depicted on the right. The box shows the DNase I hypersensitive site.

binding sites for nuclear factors, related (at least one of them) to transcriptional elements, in the human genomic region which is replicated in the first minute of S-phase is consistent with the relationship between initiation of replication and activation of transcription, which has been previously reported (Laskey *et al.*, 1989; Bird, 1986).

This might explain why several replication origins contain promoter elements, or bind transcription factors, or are close to transcriptional promoter (Umek *et al.*, 1989). On the other hand, several lines of evidence indicate that in mammalian cells, the gene expression program can influence the timing of DNA replication along chromosomes and viceversa (Dhar *et al.*,1989; Holmquist, 1987).

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