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SEQUENCE-SPECIFIC DNA BINDING PROTEIN(S) THAT BIND(S) TO A PUTATIVE HUMAN DNA REPLICATION ORIGIN

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The replication of eukaryotic genomes occurs by the activation of multiple tandemly organized replication units (replicons) each containing a bidirectional origin[1]. Somatic animal cell chromosomes contain several thousand replicons (about one per 50–100 kb on the average). The key constitutive element of a replicon is the origin which, by analogy with the prokaryotic equivalent, is thought to be a specific nucleotide sequence where DNA replication starts and where the control mechanisms must exert their action. Origin activation probably requires a critical sequence of preceding events, a proper dosage of regulatory factors and a suitable chromatin structure. More and more evidence has been accumulating in favour of a correlation between replicative and transcriptional controls. It is also possible that an active origin is not simply a nucleotide sequence but and epigenetic complex (sequence plus structure) such as that envisaged by the attachment of DNA to some chromosome support elements (scaffold, nuclear matrix, etc).

Cloning of putative replication origins. Since a large number of experiments performed in different labs indicated that cloned animal cell DNA origins are inactive when re-introduced into the same cells[2], we devised a method whereby presumptive origin sequences are isolated on the basis of their being replicated at the onset of the S-phase. The method was based on: a) synchronization of human HL60 cells at the G1/S border by a double block with aphidicolin; b) release from inhibition and labeling of newly synthesized DNA with ^{3}H-BrdU; c) separation of heavy, newly synthesized DNA, by repeated CsCl density gradients; d) cloning of DNA fragments into a suitable vector. Most of the ^{3}H-labelled DNA banded at heavy-light (HL) density, however a fraction of heavy-heavy (HH) DNA was also present, possibly due either to reinitiation from the same origins or to extrusion of nascent DNA molecules in the double stranded form due to branch migration in sheared DNA. DNA from both fractions were cloned into pAT153; 67 recombinant clones were obtained form HL DNA and 158 from HH DNA; the size of the inserts ranged between 200 and 1500 bo with and average of 400 bp.

Features of cloned newly synthesized DNA. The analysis of the entire collection of fragments showed that: a) there are no extensive homologies among different fragments; b) the sample is not significantly enriched for highly repeated sequences; c) the sample is significantly enriched in rapidly renaturing DNA (snap-back DNA, $C_0t=0$); d) most of the longest fragments behave as single copy DNA in Southern blot hybridization with genomic DNA.

Features of pB48. The largest of the clones obtained (pB48) was confirmed to be replicated exclusively during the first hour (1/8) ot the S-phase by Southern blot hybridization. The fragment is 1560 bp long and was entirely sequenced. The nucleotide sequence of this plasmid shows several interesting features that could indicate a possible regulatory role. The last 189 nucleotides correspond to one element of the human *Alu* family. At least three, thermodinamically stable stem-loop structures can be envisaged. The first one contains two possible Sp1 factor binding sites. Two A-homopolymers encompass the third stem-loop structure, which contains homologies to the SV40 and human Ig (k-chain) enhancers (70% homology), and to the region of the central palindrome in the origin of replication of the human papovavirus JCV (70%). Two more regions homologous to the same JCV origin sequence are found in the first and the second palindromes (100% and 78% homology, respectively). In addition, in the same region of the third loop, there is a 83% homology to a nuclear

matrix associated DNA sequence. Lastly, an AT-rich region after the third palindrome shares extensive similarities with the putative regions of anchorage to the chromosomal scaffold mediated by the topoisomerase II[3].

Sequence Specific binding factor. As pB48 appeared to be rich of homologies with known regulatory signals, we looked for possible binding sites for specific nuclear factors by a Band Shift Assay[4]. The incubation of a central 521 bp *AvaI -AvaI* fragment (nucleotides 445-965) with both HeLa and HL60 nuclear extracts[5] caused the appearance of retarded bands.

The effect of the two extracts is qualitatively similar except that the HL60 extract seems to produce a ladder of minor bands whose nature still has to be defined. The 521 bp fragment was then cut at a *BglI* site into two labeled fragments of 326 and 195 bp that were submitted to the Band Shift Assay under different buffer conditions (Fig 1, Panel A). Only the 195 bp *BglI-AvaI* fragment shows retarded bands while the other one is unaffected under any conditions. Maximum shift was observed in the presence of 5 mM Mg^{++}. In the absence of Mg^{++} two weaker bands are visible that migrate at higher positions. The proof that the new bands represented specific complexes was obtained by performind binding reactions in the presence of varying amounts of cold homologous competitor DNA (plasmid pB48, linearized) and non homologous DNA (vector alone). As shown in Fig. 1, Panel B, a 20 fold molar excess of the cold 195 pb *BglI-AvaI* fragment almost completely abolishes the complex formation (lane 6), while the same excess of heterologous DNA has no effect (lane 7). These data clearly indicate that the 195 bp fragment contains a recognition sequence for one or more nuclear factors.

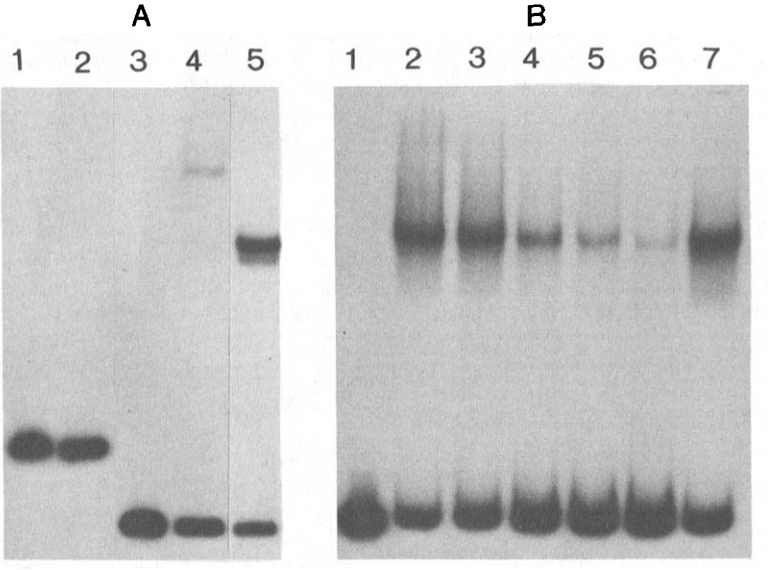

Fig. 1 - **Binding of nuclear factor to pB48 DNA** Panel A: Band Shift Assay on two adjacent fragments: the 326 *AvaI-BglI* fragment (nucleotides 445-770) (lanes 1-2) and the 195 bp *BglI-AvaI* fragment (771-965) (lanes 3-5). Standard buffer (SB): 20 mM Hepes, pH 7.3, 50 mM NaCl, 0.2 mM EDTA, 5% glycerol. Lanes 1 and 3: without nuclear extract; all other lanes: with Hela nuclear extract. Lanes 2 and 5: SB plus 5 mM $MgCl_2$ Panel B: Binding Competition Analysis. Lane 1: pB48 *BglI-AvaI* fragment alone. Lanes 2-5: after incubation with HeLa NE. Lanes 3 to 6: plus 65, 130, 260 and 520 ng respectively of pB48 plasmid (2.5, 5, 10 and 20 fold molar excess). Lane 7: plus 500 ng of vector (pAT153).

In order to determine the nucleotide sequence of the binding site, the 195 bp fragment was submitted to Footprint Analysis with partial DNase I digestion[4]. The results clearly indicated a protected region of 17 nucleotides on the retarded band between nucleotides 801 and 785. The corresponding sequence is the following: 5' TT**CGTCACGTGATG**CGA 3'. It is worth noting that the 12 central nucleotides of this sequence are palindromic except for one mismatch.

The above mentioned features of pB48 make it a strong candidate for a role in initiation of DNA replication in human cells. Purification and characterization of the nuclear factor(s) that bind(s) to the described sequence are in progress.

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