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Characterization of a human DNA sequence which replicates at the onset of the S phase

Thesis submitted for the degree of
"Magister Philosophiae"

CANDIDATE
Francesca Demarchi

SUPERVISORS
Prof. Arturo Falaschi
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October 1990

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CONTENTS

1. INTRODUCTION

This thesis is about the problem of the origins of DNA replication in animal cells and focuses in particular on two aspects of this problem: the correlation between replication and transcription, and the nuclear localization of the origins of DNA replication.
The Introduction contains an overview of the available informations on the problem as well as an introduction to the experimental part of the thesis. The experimental work presented is about the characterization of an early replicating human DNA sequence. Some preliminary conclusions are drawn and some proposal for the future work are advanced in the Discussion.

1.1 ORIGINS OF DNA REPLICATION IN ANIMAL CELLS

A full understanding of the process of DNA replication in animal cells requires the identification and characterization of replicons and their *cis*-acting control elements. Although bacterial and viral replication origins have been isolated and their activity studied *in vivo* and in *vitro*, analysis of eukariotic genomic origins has been hampered both by the complexity of replication in these biological systems and the lack of appropriate assay techniques for measuring origins function.

Much of our knowledge about the organization and operation of eukariotic origins has come from studies on the yeast *Saccaromyces cerevisiae*. Using the ability of this organism to

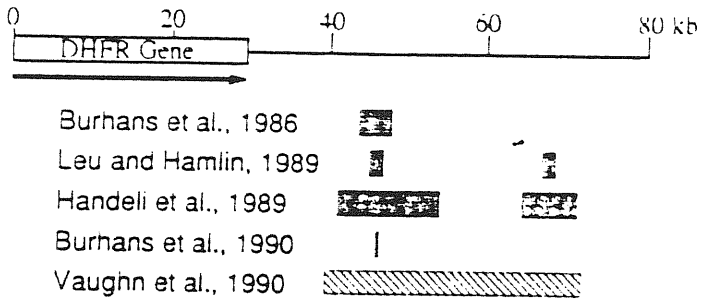
support extrachromosomal replication, it has been possible to identify numerous chromosomal DNA elements that can serve as autonomous replicating sequences, ARSs (Struhl (79)). Although this does not constitute proof that these same sequences function as chromosomal origins, several lines of evidence suggest that this is probably the case. The best example is the well characterized ARS element within the ribosomal DNA repeat unit, whose activity as an endogenous origin can actually be visualized by electron microscopy (Saffer (86)). More generally, by taking advantage of the structural features in the region of a replication fork, it has been possible to develop a two dimensional gel electrophoresis assay to detect origins in action (Brewer (87)), (Huberman (87)). Analysis by this technique has shown conclusively that some, but not all, ARS elements are used as chromosomal origins *in vivo*.(Umek (89)).

1.2 THE DHFR REPLICON

Fiber autoradiography studies demonstrate that animal cells replicate most of their DNA from multiple bidirectional origins spaced at intervals of 50-330 Kb (Edemberg (75)). The characterization of specific origin elements, however, has progressed slowly, and relied heavily on relatively indirect methods that are applicable to very early replicating domains.

The replicon which has been more extensively studied is the dihydrofolate reductase (DHFR) gene region, which has been amplified over 1000-fold in some cell lines. Using the DHFR amplicon, it has been possible to identify those DNA fragments that are the very first to be labelled following entry into S phase; these segments are presumed to overlap the region containing the actual origin element (Heintz (82)). This approach has been used to pinpoint two origins located downstream of the DHFR transcription unit (Heintz (83)), (Burhans (86)), (Leu (89)). These same

sites have been confirmed by strand exclusion, a method that employs cross-linking to distinguish nascent DNA present within the region of replication initiation sites (Anachkova (89)) and replication polarity analysis, a general method for assaying the *in vivo* direction of replication by hybridizing the newly replicated fragments of the leading strand with a set of strand specific probes encompassing the origin region (Hendeli (89)). An independent technique, which measures Okazaki fragment polarity, has been recently developed and used to map the left hand origin of the DHFR gene to even higher resolution, within 450 bp (Burhans (90)). Recently the two dimensional gel analysis technique developed for the yeast system has been applied to the DHFR amplicon to precisely map the initiation sites. The results confirm that DNA replication starts in a specific region; however they suggest that replication can initiate anywhere within a 28 Kb zone containing the two putative origins previously identified (Vaughn (90)). The diagram below summarizes the above mentioned results.



Origin localization near the DHFR gene in Chinese Hamster cells.

1.3 MODELS FOR THE INITIATION OF DNA REPLICATION

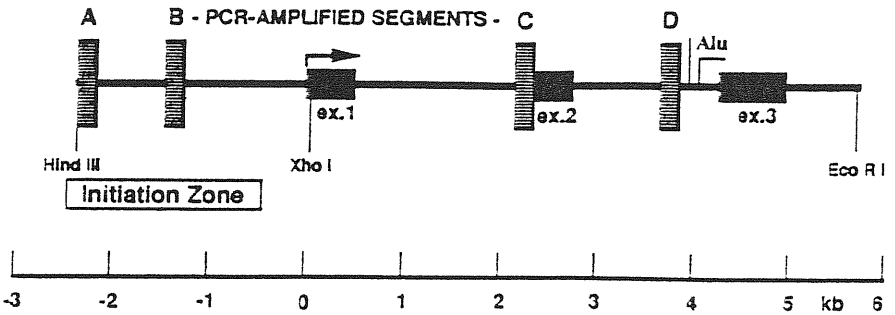
These apparently contradictory data have been produced by different techniques which probe different aspects of replicating molecules. Okazaki fragment polarity mapping identifies in which direction leading and lagging nascent strands are created on a template. As suggested in a recent minireview

of Cell by Huberman , this method may be biased toward faster stages of replication, because the efficiency of label uptake can play a critical role: long-lived replication intermediates may be poorly labeled in short pulses. In addition, newly synthesized DNA is not exclusively involved in replication; repair and recombination may also involve DNA synthesis. On the other hand, 2D gels techniques detect replication intermediates based on their abundance in a population of molecules, and therefore they are biased toward intermediates that accumulate, that is they tend to detect slower stages of replication. Moreover the detected intermediates may also be products of recombination or other processes.

On the basis of the available data, two alternative models for the initiation of DNA replication have been proposed. According to one model, supported by 2D gel analysis, initiation takes place throughout a large zone encompassing the origin of replication (Vaughn (90)). This model fits also with the data from electron microscopic studies of DNA isolated from proliferating mammalian cells which showed that molecules containing clusters of bubbles of several hundred base pairs are much more abundant than molecules containing larger bubbles or forks. One possible explanation for the requirement of a large zone of initiation is that packing hundreds of origins into small regions of the nucleus imposes structural constraints on parental strand unwinding that can only be resolved by strand unwinding at multiple sites throughout a long zone (Laskey (89)). An alternative model proposes that initiation occurs at a unique site in the replicon as it is the case in the very well studied prokariotic, viral and yeast systems. The above mentioned mapping of the switch site of Okazaki fragments polarity at the DHFR locus (Burhans (90)) is the latest and strongest argument in favour of this second model.

1.4 ANIMAL ARSs

Using the functional assay developed for the yeast to isolate ARSs, genomic DNA elements capable of supporting autonomous replication in animal cells have also been identified (Iguchi-Ariga (88)), (Frappier (87)); however in only one case, the c-myc gene upstream region, there is independent evidence showing that this same sequence serves as an origin within the chromosome (McWhinney (88)). The first reports have been recently confirmed by mapping an origin of chromosomal DNA replication in a 2 Kb region upstream of the c-myc proto oncogene in Hela cells using a PCR based method (Vassilev(90)). Moreover the initiation region has been mapped to a higher resolution by bisecting the original c-myc insert which confers autonomous replication to the plasmid vector in a transient replication assay (Mc Whinney (90)).



Origin localization near the c-myc gene in HeLa cells [Mc Whinnie (90)]

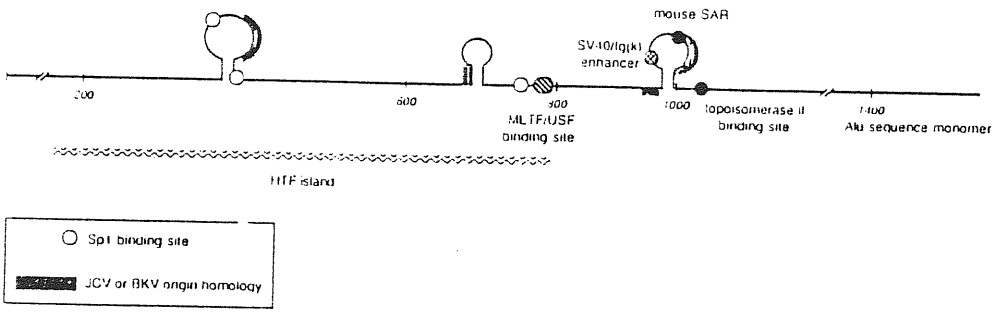
1.5 PHYSICAL METHODS FOR ISOLATING ORIGINS OF REPLICATION

Since the c-myc upstream region rapresents the only animal sequence so far characterized capable of conferring autonomous replication to a plasmid vector in a transient replication assay, at the moment the only practicable approaches to isolate origins of DNA replication are those based on the enrichment of such sequences by physical methods. In an attempt to set up an original method for the enrichment of origins of replication, we decided to check whether the pulse field gel electrophoresis technique could be used for this purpose. The experimental strategy and the result will be discussed in the results section.

Some years ago, in our laboratory, an approach based on the synchronization of cells at the G1/S border and purification and cloning of the DNA synthesized at the onset of the S phase has been followed and such DNA has been cloned from HL60 cells (Tribioli (87)). A detailed analysis of the longest fragment, pB48 (1560 bp) has been undertaken and is still in progress. In order to investigate a larger chromosomal region containing this fragment, we isolated from a human placenta DNA library a 13.7 Kb clone whose location was subsequently mapped on chromosome 19 by in situ hybridization. Hybridization experiments with DNA replicated at different S-phase intervals using this region as a probe proved that it is indeed completely replicated within the first two minutes of the the S phase.

1.6 PB48 REPLICON

An array of fragments of this genomic region was used as probe for hybridization to human DNA extracted at intervals after entry into S phase in synchronized cells in an experiment planned to follow the actual movement of the replication fork in the living cells. Preliminary results suggest that a replication origin is present within a 1 kb fragment and fires bidirectionally.The analysis of this early replicating sequence revealed the presence of homologies with the known replication origins of human Papovaviruses, recognition sites for transcription factors and putative regions of attachment to the chromosome scaffold. These last two features are common to other characterized eukariotic origins of DNA replication and it is the major aim of these thesis to address the question of the meaning of the presence of this signals in the context of DNA replication origins.



1.7 POLARITY OF REPLICATION

Close relationship between DNA replication and transcription is generally observed both in prokariotes and eukariotes. In prokariotes, transcriptional activation of replication is observed in λ phage DNA replication (Furth (83)), (McMacken (88)) and E. coli chromosomal replication (Baker (88)). Moreover, the relative orientation of replication forks and transcription units is fundamentally important for survival of *Escherichia coli*, RNA polymerase on most highly transcribed genes travel in the same direction as replicating DNA polymerases (Brewer (88)).

The same problem could be a major factor in determining the positions of many initiation sites of replication within eukariotic chromosomes. Smithies (82) has proposed that the transcriptional potential of a gene may be directly related to its replication polarity. According to this hypothesis, active genes are replicated from upstream origins, whereas inactive genes are replicated from either upstream or downstream origins. In agreement with this model, it was shown that a number of active genes are replicated in the transcriptional direction, for example the avian alpha-globin genes alpha-pi and D (James (86)), the extracromosomal rDNA genes of Tetrahymena thermophila (Ceck (81)). A further evidence which supports this model is the demonstration that the active H5 gene is replicated from an upstream origin in its 5' flanking DNA, whereas the inactive H5 gene are replicated from downstream origins (Trempe (88))

shorter S phases of embryos are largely achieved by initiating replication at closer intervals on the DNA. A possibility is that the close spacing of replication forks in early embryos can only be achieved on transcriptionally inactive templates and that the longer spacing of replication forks in transcriptionally active cells is required to coordinate the traffic of RNA polymerase and DNA polymerases on the same DNA template. This interpretation might help to explain why transcriptionally quiescent *Xenopus* eggs are able to replicate injected foreign DNA without regard to its sequence. In addition, it might explain why several replication origins contain promoter elements, or bind transcription factors, or are close to transcriptional promoters (De Pamphilis (88)).

1.10 *CIS* ACTING TRANSCRIPTION SEQUENCES AT THE ORIGINS OF DNA REPLICATION

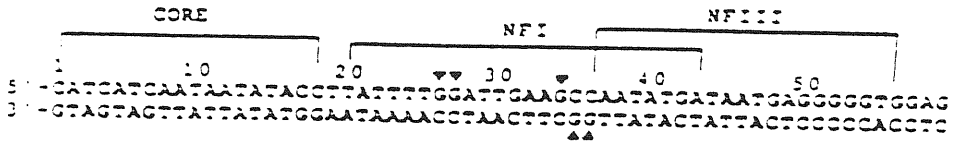
In the light of the available data on animal cells systems and by analogy with other simpler and better understood systems, it is reasonable to assume that replication origins must contain at least three elements (Linskens (90)): an origin of bidirectional replication (Burhans (90)) where Okazaki fragments switch polarity, the initiation zone required for initial unwinding and strand synthesis events (Vaughn (90)) and the sequence recognized by initiation proteins sometimes referred to as *ori.*

All three elements are confined within few hundreds nucleotides in the origins of *E. coli,* SV40 and yeast, but this is not necessarily the case in animal cells. This possible difference could help to explain the almost complete failure in isolating ARSes in animal cells.

1.11 TRANSCRIPTION FACTOR BINDING SITES AT VIRAL ORIGINS

Activation of DNA replication by enhancers and other transcription elements appears to be a quite general feature of the replication of eukariotic viruses. In the following chapter the available data relative to Adenovirus and Papillomavirus are presented.

ADENOVIRUS



Sequence of the adenovirus type 2 origin of DNA replication

Binding of transcription factors is clearly documented for replication of adenovirus DNA, a system that can be reconstituted *in vitro*. Replication of human adenovirus DNA occurs by a protein-priming mechanism in which a precursor of the viral terminal protein becomes covalently bound to the first nucleotide, a dCMP residue, thus forming a PTP-dCMP initiation complex. The 3' OH group of dCMP serves as a primer for further polymerization by displacement mechanism (Stilmann (89)). Initiation can be reconstituted *in vitro* and minimally requires the complex of the viral DNA polymerase and the viral terminal protein and the core origin consisting of nucleotides 1-18.

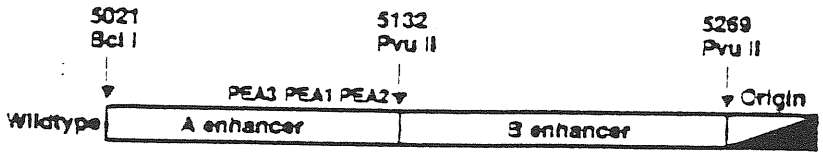
Initiation of DNA replication is considerably enhanced by two cellular proteins, nuclear factor I (Nagata (82)), (Rosenfeld (86)) and nuclear factor III (Pruijn (86)), (Rosenfeld (87)). These proteins

bind to the auxiliary region of the origin located between nucleotides 25 and 50. NFIII was purified from HeLa cells as a 90-95 Kd protein which is indistinguishable from the ubiquitous octamer transcription factor OTF-I (O'Neil (88)). This protein, also called OCT 1, OBP or NF-A1, binds to the octamer element; 5'-ATGCAAAT-3' (Singh (86)) , (Sturm (87)). This element is an essential component of promoters and enhancers of cellular genes as diverse as histone H2B, U1 and U2 snRNAs and immunoglobulin light and heavy chains.

NFI from HeLa cells is indistinguishable from the CCAAT-binding transcription factor (CTF) and consists of a family of polypeptides of 52-66 Kd. The CCAAT binding sequence is an essential promoter element of many mRNA coding genes (Benoist (80)).

To address the question of the mechanism for activation of DNA replication of these transcription factors deletion analysis has been carried on. It was shown that the transcription activation domain is dispensable for replication activation (Mermod(89)), (Verrijzer (90)). This result suggests that the mechanisms for activation of DNA replication and transcription differ. One possible explanation is that binding itself is sufficient , leading to structural change in the origin which enables efficient formation of an initiation complex, for instance by providing access to other viral replication proteins. Alternatively, the DNA binding domain could also contain sites for direct protein-protein contacts with other replication factors leading to the formation of an initiation complex or to the stabilization of such a complex.

POLYOMAVIRUS



Organization of the origin of DNA replication of Polyoma virus.

Polyomavirus (Py) DNA replication is a model for cellular DNA replication, as it closely resembles that of the host cell in many respects. Binding sites for HeLa nuclear transcription factors are present adjacent to Py origin of replication, A and B enhancer. Either one can drive replication or transcription in 3T6 or 3T3 cells (O'Connor (88)). The A enhancer has a binding site for PEA1, the murine homolog of the human AP1 trans acting factor (Martin (88)). AP1 appears to be important for several regulatory processes of the cell, including transcriptional regulation by phorbol ester, TPA (Angel (87)), EGF and serum (Franza (88)), the ras cell surface signal transduction pathway (Ingler (88)), cell cycle control (Ryseck (88)), cellular transformation (Bohman (87)) and terminal differentiation of F9 cells (Chiu (88)). Furthermore, AP1 appears to act in conjunction with other factors and can be found physically associated with c-Fos protein (Chiu (88)).

Minimal enhancer elements have been defined both for transcription and replication . Py DNA replication is fully activated when the PEA1 binding site is present in 2 copies (52 nucleotides), whereas 7 tandem copies (182 nucleotides) are required for full transcriptional enhancer activity (Rochford (90)).

An interesting point is that *in vitro* DNA replication systems which utilize naked Py DNA templates have not yet shown a requirement for an origin-proximal enhancer (Prives (87)). This implies a role for a specific DNA-chromatin protein structure in enhancer mediated replication control. As for a possible function

for PEA1 (AP1) and PEA3 transcription factors in Py DNA replication, it is tempting to think that they could play a role in the determination of cell specific DNA replication. If this is true the understanding of the Py system could also shed light on the regulation of cell type specif replication of the more complex animal chromosomes.

1.12 A DNA REPLICATION ENHANCER IN *SACCHAROMYCES CEREVISIAE*

All ARSs show a common 11 bp (A\T)TTTAT(A\G)TTT(A\T), core consensus sequence essential for origin function. Nucleotide sequence on either side of the core consensus are required, although the amount and the sequence of the flanking DNA vary depending on the ARS (Stinchomb (79)).

Recently, a protein which specifically binds to a DNA sequence present in a broad spectrum of origins has been isolated (Sweder (88)), (Diffley (88)), (Halfter (89)), and the corresponding gene cloned (Halfter (89)), (Rhode (89)). This protein, which is known with a number of different names: ABFI, OBFI, BAF, SBFI it is also a transcriptional activator of many yeast genes and functions as a repressor in conjunction with RAPI at the HMRE silencer.

Using *in vitro* site directed mutagenesis, the autonomous replicating sequence ARS 121 which contains two OBFI binding sites has been dissected (Walker (90)). Three domains appear to be important for origin function; the first one contains the ARS consensus, the second one contains several elements with near matches to the core consensus, and the third one contains two OBFI binding sites. Interestingly enough a synthetic OBFI DNA binding site can substitute for the entire third domain for origin function. Moreover stimulation of *ori* function by the synthetic binding site is orientation independent and can occur at a distance as far as 1 Kb upstream to the essential domain.

These results lead to the conclusion that the OBF I binding site is an enhancer of DNA replication and OBFI protein is likely to be involved in the regulation of the activation of nuclear origins of DNA replication in *S.cerevisiae*.

Organization of the ARS 121. I and II are OBF I binding sites. 1, 2, 3 are close matches to the core consensus. 4 is the core origin.

1.13 TRANSCRIPTION SIGNALS IN PB48

The early replicating sequence pB48, isolated in our laboratory from HL60 cells, is rich in transcription signals . It contains a 600 base pairs HTF (*Hpall* Tiny Fragments) island typically present upstream of house-keeping genes, three putative binding sites for spl transcription factor and one sequence with 70% similarity to the SV40 enhancer. Moreover, it contains a 17 base pairs sequence, 5'-TTCGTCACGTGATGCGA-3' that binds to a nuclear factor, as shown by band shift and footprinting experiments.

In order to assess the promoter activity of this region, CAT assay experiments have been performed. By this mean we showed that a fragment containing pB48 in the appropriate orientation is able to promote transcription of the downstream reporter gene. Moreover, we showed that a region of 100 base pairs containing the above mentioned binding site and the TATA box is sufficient to promote transcription in a CAT assay.

The protein binding sequence mentioned above is highly homologous to the upstream element of the Major Late promoter of Adenovirus (Miyamoto (84)), (Shi (86)) and to the upstream elements of different cellular genes: the rat γ fibrinogen gene (Chodosh(87)), the mouse metallothionein gene (Cartew

(87)), the human growht hormone gene (Paritz (88)), the gene Hox 1.1 of mouse, Xenopus (Kessel (88)) and man (Simeone (87)). Furthermore similar sequences are found in the yeast centromer element CDEI and in the GAL 2 promoter, in the TRP1-ARS1 plasmid and in the HIV 1 Long Terminal Repeat.

The cellular protein which binds to the upstream element of MLP has been purified in different laboratories and called MLTF (major late transcription factor) (Cartew (85)), or USF (upstream stimulatory factor) (Sawadogo (88)).

To answer the question whether there is a correlation between the transcription signals of pB48 and replication, a series of experiments, that will be described in the results session, have been performed.

1.14 REPLICATION OCCURS AT NUCLEOSKELETON

In eukariotic cells, chromosomal DNA appears to be organized into looped domains by periodic attachment to a proteinaceous nuclear structure that is commonly referred to as the nuclear matrix or scaffold (Nelson (86)). The loops, which are topologically constrained, are comparable in size to the average eukariotic replicon (Volgelstein (80)).

Both transient and permanent attachment of DNA to the nuclear matrix have been suggested. Transient attachment appears to occur at replication forks, since nascent DNA has been found to be closely associated with the matrix (Dijkwel (79)), (Jackson (86)). The presence of DNA polymerase α (Smith (80)), DNA primase (Wood (86)) and topoisomerase II (Berries (85)) in nuclear matrix preparations further supports this hypothesis. Transient attachment of DNA may also occur during transcription, since nascent RNA is observed to partition with the nuclear matrix (Jaekon (81)).

Permanent matrix attachment regions (MARs) may also be involved in different functions. Several lines of evidence suggest that MARs are close to replication initiation sites in mammalian cells. When replicating nuclei are pulse labelled with either BrdUrd or biotynilated dUTP, sites of replication can be visualized microscopically. Replication is confined to a few hundreds of discrete foci each of which must consist of several hundreds replication forks. (Nakayasu (89)), (Nakamura (86)). In yeast a subset of chromosomal ARSes appear to associate with the nuclear scaffold (Amati (88)).

A second type of permanent attachment occurs at or near *cis*-acting elements involved in the regulation of gene expression. For instance, a MAR is present upstream of the κ immunoglobulin enhancer in murine cells (Cockerill (86)) and in the enhancer-like sequence located upstream of three developmentally regulated genes in Drosophila (Gasser (86)).

In the DHFR amplicon of the metotrexate resistant CHO cell line, CHO400 permanent attachment sites are found between the two close initiation zones upstream of the DHFR gene and near a junction between amplified units (Dijkwel (89)).

Since both replication forks and origins of DNA replication have been shown to be attached or situated very close to the nuclear scaffold it is possible that both initiation and elongation may proceed at fixed sites in the nucleus (Nakamura (86)). Moreover, since topoisomerase II has been suggested to mediate anchorage of DNA to the nuclear matrix, it is tempting to speculate that this protein may recognize DNA sequences involved in the initiation of DNA replication (Amati (88)), (Gasser (86)). The topoisomerase II/origin complex at an initiation site could then serve as a nucleation center for the formation of a larger multienzyme complex which then carries out the elongation phase of DNA replication (Reddy (83)). With this in mind, we decided to study the interaction of our early replicating sequence and the topoisomerase II *in vivo*.

2. RESULTS

2.1 PFGE: A TRAP FOR REPLICATION FORKS ?

Pulse field gel electrophoresis (PFGE) is a technique that permits the physical separation of small and large DNA fragment with theoretically no upper size limit. PFGE subjects DNA to field pulses applied at an angle of greater than 90 degrees to each other. Separation probably results from the differing abilities of molecules of different length to reorientate to the changed field direction (Lai (89)).

It has been suggested that replication forks, due to their topological complexity, do not behave as linear molecules in a PFGE. (Smith (86)). We decided to check this hypothesis using the human early replicating sequence pB48 as a probe.

Human HL60 lymphoblastoid cells were synchronized at the G1/S border with aphidicolin and aliquots were taken at different intervals after entry into S phase. Each aliquot was used to prepare agarose plugs and digested with the rare cutter restriction enzyme *SfiI* as described in 'Material and methods'. The plugs were run in a PFGE apparatus, the gel was transferred onto a nylon membrane and hybridized with a ^{32}P random primed - labelled pB48 probe.

The expected result was the detection of a retarded band in the well loaded with DNA from early S cells compared to the DNA from G1/S cells. On the contrary , as reported in figure 1, panel II, this was not the case. In the conditions used the working

hypothesis failed to be proved; however to rule it out we should repeat the experiment using a set of different probes encompassing the early replicating region.

2.2 A PROMOTER IN THE EARLY REPLICATING HUMAN DNA SEQUENCE PB48

We already showed that a 900 bp pB48 insert in the appropriate orientation can promote transcription from a downstream reporter gene in a transient transfection assay (Falaschi (88)). To map this promoter to a higher resolution, we cloned in the assay plasmid, pCAT0 a pB48 fragment of 100 base pairs in both orientations with respect to the CAT reporter gene.

As it is shown in figure 2 , the 100 base pair fragment is sufficient to drive transcription of the downstream reporter gene, when present in the appropriate orientation, even if at a lower extent than the longer fragment. The fragment sufficient for promoter activity contains a TATA box and the binding site for a cellular protein, as previously showed by band shift and footprinting experiments (Tribioli (87)).

2.3 MORE THAN ONE PROTEIN BIND TO THE PB48 BINDING SITE

By means of band retardation assays it was shown that the pB48 sequence contains a binding site for a nuclear factor present both in the HeLa and HL60 cells (Tribioli (87)). Further experiments demonstrated that the binding site of pB48 is similar to the upstream element of the Major Late Promoter of Adenovirus 2 and that both sequences compete for the binding to the same factor.

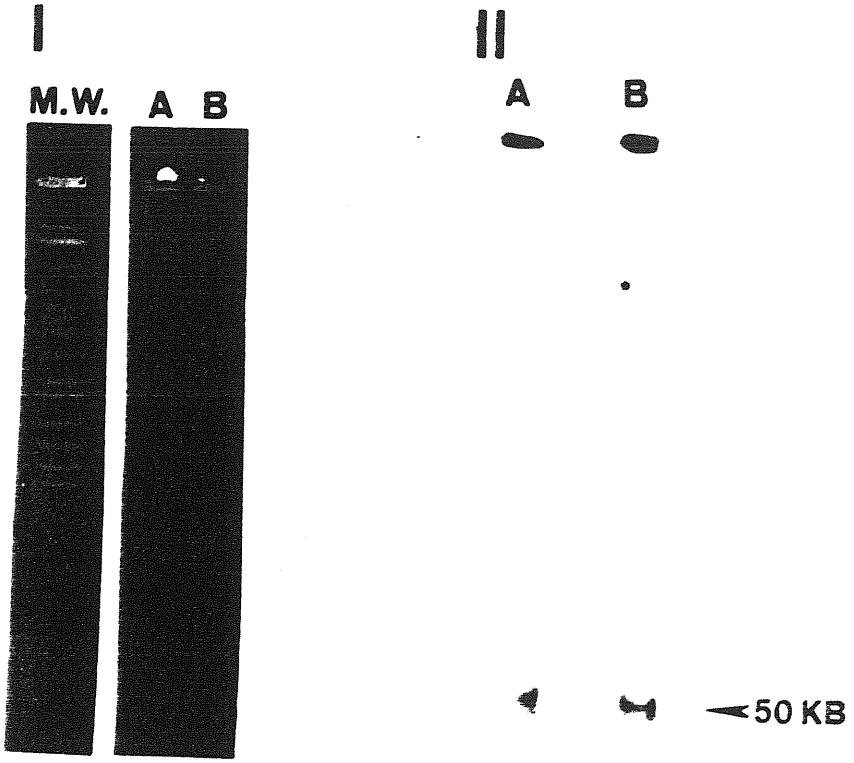


Figure 1. Panel I : Pulse Field Gel Electrophoresis. From left to right: *S. cerevisiae* chromosomes (M. W.), G1/S HL60 DNA digested with *Sfi I*, early S HL60 DNA digested with *SfiI* .

Panel II : Southern blot of the PFGE of panel I

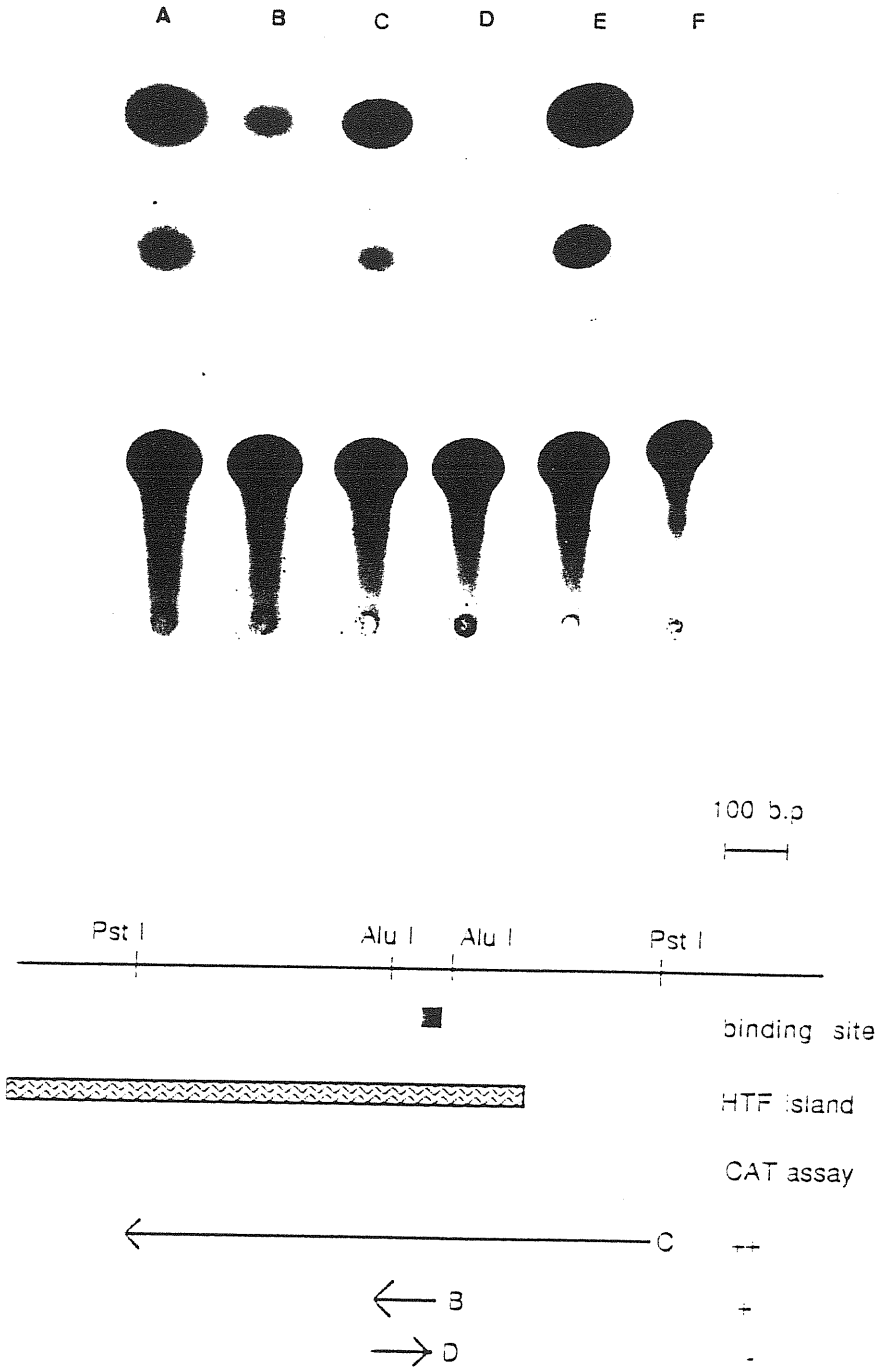


Figure 2: CAT assay and schematic rapresentation of the results. A (*E. coli* CAT enzyme) and E (pSV2 CAT) are positive controls, F is a negative control (pCAT 0).

The analysis of the binding specificity for nuclear proteins to pB48 was extended by performing a Southwestern experiment. Briefly, a HeLa cell nuclear extract was resolved on an SDS-polyacrilamide gel, soaked in an urea containing buffer to remove SDS and transferred onto nitrocellulose membrane. After preincubation in a 5% nonfat dry milk buffer, which minimizes nonselective DNA proteins interactions, the membrane was probed with pB48.

As shown in figure 3, three polypeptides of 44, 70 and 110 Kd respectively are specifically recognized by the probe. The 44 Kd polypeptide most likely corresponds to the protein (MLTF/USF) that was described to bind Ad2 MLP and to activate transcription (Sawadogo (88)). The 110 Kd polypeptide could correspond to the 116 Kd protein that was recently demonstrated to interact with the Adenovirus 2 MLP by UV dependent cross-linking experiments (Safer (88)).

2.4 BINDING COMPETITION EXPERIMENTS

Binding sites for the same activity that binds to pB48 are found in the Adenovirus MLP as well as upstream of a number of eukariotic genes. Competition for binding of the factor(s) to the pB48 DNA sequence in gel retardation assay was used to identify other possible target sequences both in the human and in other eukariotic genomes.

Band shift competitions were performed by incubating a ^{32}P labelled 24-mer oligonucleotide encompassing the binding site of pB48 with an excess of cold competitor DNA. Molar excess of 10, 20, 40 fold were used when the competitor was a restriction fragment, while higher molar excess (20, 60, 180 fold) were used with cold oligonucleotides competitors. The affinity of the nuclear factor, indeed, was lower for oligonucleotides than for longer DNA

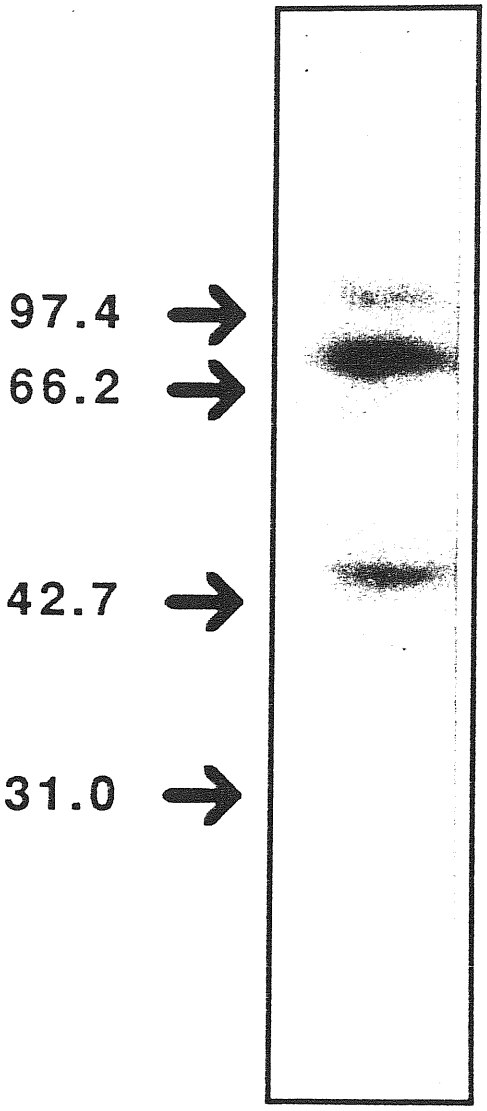


Figura 3 : South-Western blotting. Probe: oligonucleotide pB48

fragments, in agreement with what recently observed for USF (Sawadogo (88)), probably due to the involvement of surrounding non specific DNA sequences in protein - DNA interactions.

The results of competition experiments with a variety of sequences are shown in figure 4. The strength of competition is indicated by (+/-) symbols when the competition assay was performed with a purified cold restriction fragment, or is visualized by the intensity of the retarded band in the case of oligonucleotides. Competition was observed with a fragment containing the MLP of Ad2 and an oligo corresponding to its upstream sequence, with a fragment of HIV-LTR as well as with an oligonucleotide encompassing nucleotides 174 to 151 upstream of the transription start site of viral DNA. An insert containing CDEI and CDEIII of yeast CEN VI competed poorly for binding, while a clear competition is observable when an oligo for region CDEI is used as a probe. This discrepancy is probably due to the fact that the centromer fragment used contains binding sites for other human proteins which can interfere with the binding activity under study.

2.5 THE PROTEIN WHICH BINDS TO PB48 IS PRESENT IN REPLICATING AND NON REPLICATING CELLS

As discussed in the introduction, many specific DNA binding proteins are involved both in transcription and replication. The binding site described above is found nearby a putative origin of replication and is therefore tempting to think that it could function in the initiation of replication process. To test this idea we decided to study the presence of the factor in nuclear extracts in growth arrested cells. We chose WI38 human fibroblasts as a system because this cell line can be easily be pushed into the quiescent state. We prepared nuclear extracts from growing and serum deprived, growht arrested WI38 cells. The nuclear extracts were used for a band retardation assay using a labelled

Source	Sequence	Competition with pB48 Bindig Site	
		as plasmid insert	as oligo
			20x 60x 180x
pB48 Binding Site	TTCGTCACGTGATGCGA	++++	
Human Alu (Blur 8)	CAGATCACCTGAAGTCA	-	nd
Human 0-LTR Family	CCAGACATGTGGAAGTA	-	nd
Human Hox (HHO.c1.95)	GGAATCTCGTAAAACCG	-	
Mouse Hox 1.1	GCGGTCACGTGCCGCGG	nd	
Xenopus Hox 1.1	CAGATCACGTGGCCCAG	nd	
Yeast TRP1-ARS1	TTGAGCACGTGAGTATA	-	nd
Yeast Centromere (chr. VI)	TTCATCACGTGCTATAA	+	
Yeast GAL2 promoter	TGGGTCACGTGATCTAT	nd	
Adenovirus MLP	TAGGCCACGTGACCGGG	++++	
HIV-1 LTR	TTCATCACGTGGCCCGA	+++	
Human growth hormone	GCACCCACGTGACCCT	nd	nd
Rat gamma-fibrinogen	GACCCCGTGACC	nd	nd
Mouse methallotionein I	CGGGGCGCGTGACTATA	nd	nd

Figure 4 : Binding competition experiments between pB48 and other analogous sequences

oligonucleotide containing the binding site under study as a probe.

Figure 5 I shows that the retarded band due to a specific DNA protein complex is present both in growing and in G0 arrested cells. The minor band which is missing in G0 cells was subsequently shown to be due to the residual single strand oligonucleotide present in the double strand oligonucleotide preparation (figure 5 II)

2.6 TRANSCRIPTION ACTIVITY OF THE PB48 REPLICON

Transcription activity of the pB48 area can be detected *in vivo* as previously described (Falaschi (88)). When the mRNA of actively growing HeLa cells were probed with pB48 in a Northern type hybridization, two bands of 1100 and 850 nucleotides could be observed. We decided to study the transcription activity of pB48 in the cells where we know that this sequence is replicated very early. We also wanted to analyze the pB48 expression in growth arrested cells to test whether there is some relation between transcription and replication. To this end we used RNA from growing and growth arrested WI38 human fibroblasts.

Figure 6 shows the results of the Northern experiment. The region corresponding to pB48 is expressed at different levels in the different cell types analyzed. Maximal expression is found in HL60 cells from which the clone was derived and this is consistent with the fact that highly expressed genes are early replicating. The level of expression in human fibroblasts is the lowest observed, but doesn't appear to change in growht arrested cells.

Northern blot analysis, however, measures the steady state level of mRNA, thus this result doesn't rule out the possibility of a coupling between a peak of transcription activity and replication. To assess this idea run on experiments were carried on.

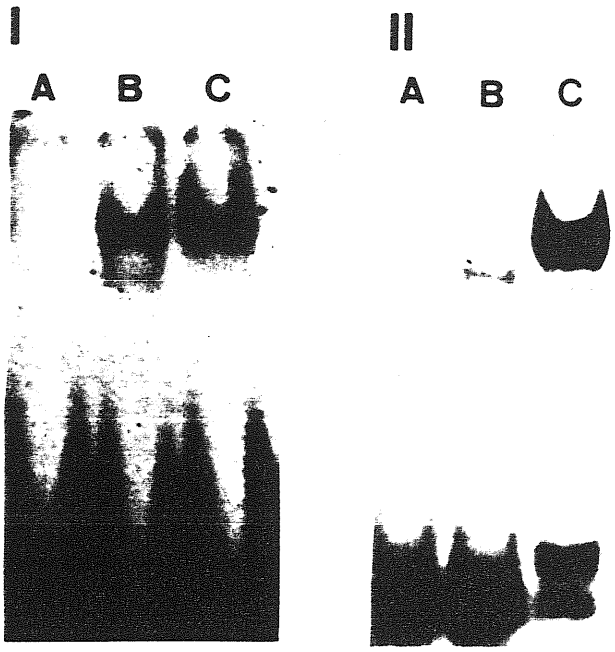


Figure 5 . Panel I : Gel retardation assay. The nuclear extracts are from growing WI38 cells (B), and from growth arrested WI38 cells (C). Lane A is the control without nuclear extract. The probe is the oligonucleotide pB48.

Panel II : Gel retardation assay. the nuclear extract is from HeLa cells. The probes are : double strand pB48 oligonucleotide (C) and single strand pB48 oligonucleotide (B). Lane A is the control without nuclear extract.

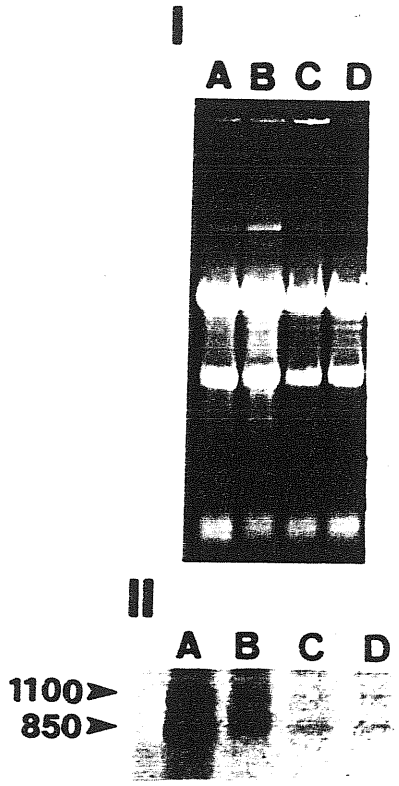


Figure 6. Panel I : Ethidium stained RNA gel used for Northern blot. A) HL60 total RNA, B) HeLa total RNA , C) growing WI38 total RNA , D) growth arrested WI38 total RNA .
Panel II : Northern blot of the gel of panel I.

2.7 IS THERE A COUPLING BETWEEN REPLICATION AND TRANSCRIPTION?

Run on assay is the technique of choice to measure the rate of transcription at a given time of the cell cycle and at a given physiological condition of the cell. We used this technique to verify whether a change in transcription activity is coupled to the synthesis of DNA at the pB48 origin of replication. HL60 cells were synchronized at the G1/S border by aphidicolin treatment and different aliquots after the release of the block were used to prepare active nuclei by NP40 lysis. The nuclei were used for nuclear run on transcription assay performed as described in 'Materials and methods' in the presence of ^{32}P dUTP. RNA from each aliquot was extracted and used as a probe against a set of dot blots. Each dot contained pBR322 DNA as negative control, γ actin and β globin cDNA -containing plasmids as positive controls and the pB48 DNA sequence under study.

As it can be seen in figure 7, the transcription activity of pB48 appears constant throughout the cell cycle.

1.8 MAPPING OF THE TOPOISOMERASE II *IN VIVO*

A large body of evidence suggest that origins of DNA replication in eukariotic cells are associated with the nuclear matrix. Topoisomerase II is a major component of the nuclear matrix and matrix associated regions of DNA contain topoisomerase II binding sites. On this basis, we decided to map the topoisomerase binding sites in the pB48 fragment *in vivo*. By sequence analysis we found a putative topoisomerase II binding site in the pB48 sequence and to test whether this site was

TRANSCRIPTIONAL ANALYSIS OF pB48 AT DIFFERENT PHASES OF THE CELL CYCLE IN SYNCHRONIZED HL60 CELLS

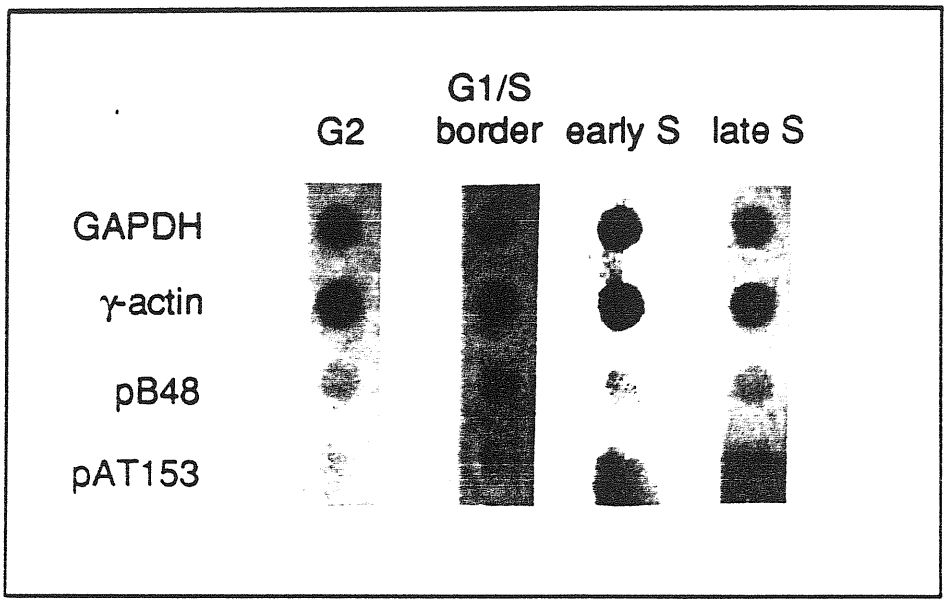
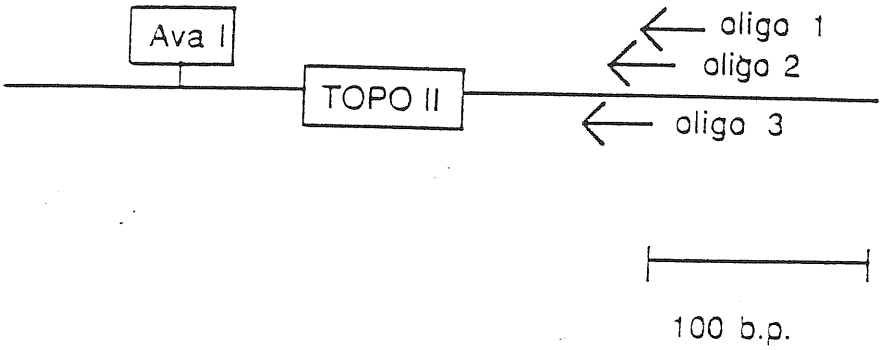


Figure 7 : RUN ON

effectively used *in vivo,* we set up a topoisomerase II mapping assay.

Catalytic sites of topo II on DNA can be detected by uncoupling the breakage and rejoining activity using detergents to denature the enzyme, thereby yielding DNA molecules with double strand breaks. The efficiency of trapping the cleavage intermediate is significantly increased through the use of drugs such as VM26 that stabilize the half life of covalent complex between topoisomerase and DNA (Chen (84)). HL60 cells were treated with VM26 and the DNA extracted with SDS. Extracted DNA was restricted with Ava I enzyme, which cleaves about 100 base pairs upstream from the putative topoisomerase II binding site, and the products were amplified by ligation mediated PCR. In the last cycle, a ^{32}P labelled oligonucleotide was added to allow the detection of products directly from a dried gel.

Figure 8 shows the results: in the negative control, lane 1, only the bands due to the restriction enzyme cleavage are detectable, in the other lane, that contains DNA from VM26 treated cells, two fainter lower molecular weight bands are visible. These bands are most likely due to topo II mediated cleavage.



pB48 region under study in the *in vivo* topoisomerase assay

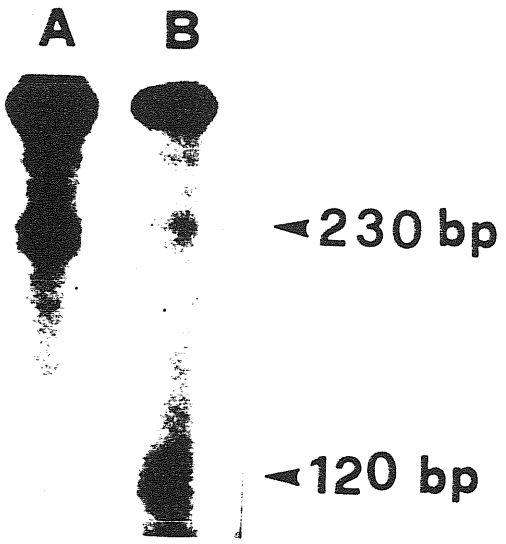


Figure 8 . Topoisomerase II assay. Lane A : LMPCR products from control cells, lane B: LMPCR products from VM26 treated cells.

3.DISCUSSION

3.1 SEARCH OF NEW TECHNIQUES FOR ISOLATING ORIGINS OF DNA REPLICATION FROM ANIMAL CELLS

The lack of appropriate assay systems to isolate origins of DNA replication has considerably slowered down the study of this central biological process in higher eukariotes. For this reason, one of the primary and more challenging goal in the field is to set up strategies to pick up origins of DNA replication. To this end we wanted to exploit the recently developed technology of pulse field gel electrophoresis. Although there have been few systematic studies on the effect of topological complexity on PFGE, it has been suggested (Cantor (87)) that replication forks could have an anomalous behavior in PFGE and we decided to verify this idea using the pB48 sequence as a model.

PB48 is an early replicating sequence in HL60 cells and preliminary results, described in the Introduction, suggest that it correspond to or is located nearby a chromosomal origin of DNA replication. If replication forks have an anomalous behavior in PFGE, a fragment of DNA extracted from non replicating cells should show a different migration mobility compared to the same fragment from replicating cells, that is the same fragment containing a replication fork. The probe used to check this idea is the pB48 sequence, the first to be isolated for its early replicating property. The negative result we obtained is just a preliminary result and we are planning to repeat the same experiment using a set of different probes from the area under study.

The failure in obtaining a positive result could have been due to the fact that in the S phase intervals studied the replication

fork was already passed or did not yet reach the DNA segment recognized by the probe.

3.2 WHAT IS THE MEANING OF *CIS* ACTING TRANSCRIPTION SIGNALS NEARBY AN ORIGIN OF DNA REPLICATION ?

One possible function of a transcriptional promoter nearby an origin of DNA replication is suggested by the transcriptional activation model proposed by Kornberg. Activation of an origin of DNA replication by transcription from an upstream promoter has been observed in λ phage and *E. coli* (Baker (88)) and it has been suggested to be due to the alteration of topological state of the origin. Transcription activation does not seem to occur in the pB48 human replicon, indeed we showed by Northern blotting and run on experiments that its transcription is constant throughout the cell cycle. This result is a further element suggesting that we are dealing with a house-keeping gene; we previously showed that pB48 is expressed in many different tissues and that the region upstream of the promoter contains an HTF island, a typical feature of housekeeping genes.

To address the question of the role of transcription signals nearby our origin of DNA replication we focused our attention on the cellular protein which binds to pB48 as shown by band shift and *in vitro* footprinting. The target for this factor is contained in the minimal region required to promote transcription as shown by CAT assay.

By means of a Southwestern experiment, we found that three polypeptides recognize an oligonucleotide containing the binding site. This result suggest that, as already reported for the octamer binding motif (muller (88)) and for the steroid receptor gene family (Evans (88)) also the binding motif described here

could be the target of different proteins (and/or different forms of the same protein). The many similar DNA sequences binding MLTF/USF related polypeptides may represent a conserved binding motif in eukariotic organisms, as already pointed out by Bran (87), possibly interacting with different factors devoted to different functions. To test the hypothesis that the pB48 binding site could play a role in the initiation of DNA replication we analyzed nuclear extracts from non replicating cells for the presence of the factor and we found that it was indeed present.

However, *in vitro* studies can be misleading because the presence of a protein doesn't necessarily mean that it is active. To clarify this point we are working on *in vivo* footprinting using ligation mediated PCR. By means of this technique, using cells from different phases of the cell cycle, we will be able to obtain a picture of the traffic of proteins in this chromosomal area.

A possible function for controlling DNA replication by transcription elements, could be the regulation of tissue-specific DNA replication. This could be an explanation for the relationship of the timing of DNA synthesis during early versus late S phase to its transcriptional activity. DNA replicated during early S phase appears to be correlated to highly transcribed DNA in specific tissues. Furthermore, the frequency at which eukariotic replicons show an association with transcriptional elements is consistent with the general involvement of cell-specific transcription factors in DNA replication. The involvement of transcriptional factors in cellular DNA replication suggests additional mechanisms by which transcriptional factors may also alter growht or transform cells by directly regulating DNA replication.

Little is known about the mechanism by which *cis*-acting elements can affect the initiation of DNA replication. It is assumed that the various transcriptional factors which bind must affect the accessibility or initiation activity of the origin to DNA replication proteins. Recently, Chang and Kelly (89) suggested that transcriptional enhancer elements present at viral origins of

replication stimulate replication by perturbing the distribution of nucleosomes in the adjacent sequences, thus leaving the origin exposed for interaction with the replication machinery. It is expected, however that such a stimulatory mechanism will be sensitive to the distance of the enhancer from the origin core sequences. Therefore this mechanism seems appropriate only for those enhancer sequences which require to be located in the proximity of the origin core. In the case of the yeast ARS enhancer, described in the Introduction, the distance from the core doesn't affect the enhancer function. In this case it seems more probable that in order to stimulate the origin of replication, the ARS enhancer and its cognate protein have to interact with either the 3' auxiliary domain or the core origin itself. This could be accomplished by DNA looping that would place the protein bound to the enhancer in proximity to a target site, enabling a direct interaction of the regulatory protein with a component of the replication apparatus. Such a mechanism has been proposed for the action of some enhancer sequences and their cognate proteins in the regulation of transcriptional activation and repression (Ptashne (86)).

The final eluciaation of the mechanism of the enhancer function in replication, however, will require the identification and characterization of the remaining components of the replication initiation apparatus.

3.3 IS THE PB48 ORIGIN OF REPLICATION ANCHORED TO THE NUCLEAR MATRIX ?

The growing evidence that eukariotic DNA replication takes place at an immobilized structural framework may explain why it has been so difficult to obtain eukariotic cell free systems that

replicate efficiently *in vitro*. In addition, it can help to explain the almost complete unsucces in isolating ARSs from animal cells.

One possible strategy to analyze scaffold-DNA interactions in eukariotic cells has been developed some years ago in the laboratory of Laemli (Mirkovitch (84)). This procedure consists in the extraction of nuclear matrix and associated DNA under physiological salt conditions and the comparative analysis of matrix bound and unbound DNA by Southern blot. By means of this approach several enhancer elements from Drosophila and mouse cells have been shown to be matrix associated (Cockerill (86)), (Gasser (86)). Moreover also a set of *S. cerevisiae* autonomous replicating sequences have been shown to be matrix associated (Amati (88)). In the DHFR amplicon of CHO400 cell line, attachment sites have been identified between the two initiation zones, upstream from the DHFR gene and near the junction between the amplified units (Dijkwel (88)).

The clustering of regulatory regions in the proximity of scaffold attachment sites appears to occur also in eukariotic viruses (Bodner (89)) and may represent a common mechanism for activation of a DNA for both replication and transcription. Therefore it is conceivable that some of the DNA sequences within that cluster serve a dual role in replication and transcription.

Topoisomerase II is one of the major components of the nuclear matrix and it has been suggested to mediate anchorage of DNA to the nuclear matrix (Wood (86)), (Gasser (86)). On this basis, we decided to map the topoisomerase II binding sites in the human DNA replicon pB48. The first step in this direction was to check whether a topoisomerase II binding site found by sequence analysis was a real target of topoisomerase II *in vivo*. We used the topoisomerase II inhibitor VM26 to trap the cleavage intermediates and amplified the extracted DNA by means of ligation mediated PCR.

The results obtained appear to confirm the binding of topo II to the site under assessment, however the same

experiment should be repeated to exclude the possibility of PCR artifacts. We are also planning to look for topo II binding sites in the larger chromosomal domain encompassing the pB48 origin of DNA replication. To this end DNA from cells treated with VM26 and from not treated control cells will be analyzed by Southern blot, after cleavage with the appropriate restriction enzymes. By comparing the electrophoretical pattern of DNA from treated and not treated cells we should be able to determine the distribution of topoisomerase II binding sites in the chromosomal region of interest. Moreover we could compare the topoisomerase II binding pattern in cells from different stages of the cell cycle. This study should provide insight into the mechanism of higher-order DNA organization and nuclear matrix binding in eukariotic DNA regulatory events.

4.METHODS

4.1 CELL CULTURE AND SYNCHRONIZATION

HL60 human promyelocitic cells were cultured in RPMI 1640 medium (Gibco) containing 50 μM/ml gentamicin, 10% fetal calf serum and 5 mM glutamine. HeLa cells were cultured in Dulbecco's modification of Eagle's minimal essential medium (DMEM) (Gibco) in monolayer culture or in Joklik's modification of Eagle's minimal essential medium (Gibco) in spinner culture, both containing 50 μg/ml gentamicin, 10% fetal calf serum and 2 mM glutamine. WI38 cells were cultured in DMEM supplemented with 10% fetal calf serum, in monolayer culture.

HL60 cells were synchronized at the G1/S border using aphidicolin (Sigma) as described (Tribioli (87)). Since synchronization causes a certain level of cell death (50-70%), the cells were layered onto lymphocyte separation medium (density 1.0077 g/ml, Boehringer Manheim) and centrifuged, and the band of cells at the interface was collected. This treatment reduced the percentage of dead cells to 25-35%. The cells were then washed twice to remove aphidicolin.

In the starvation experiments, the serum containing medium was removed and WI38 cells briefly rinsed in DMEM medium. The cells were then incubated in DMEM supplemented with 1 % serum for 72 hours.

4.2 AGAROSE PLUGS PREPARATION AND DIGESTION

The cells were washed once in sterile phosphate buffer saline and resuspended at 1X 10^6 cells per 40 µl PBS at room temperature. A 1 % solution of BRL LMP Ultra pure agarose in PBS was melted and held at 42°C, an equal volume of this was mixed with an equal volume of the cell suspension at room temperature and the agarose cell mixture was dispensed immediately in a slot former. The mould was then placed on ice for twenty min. to allow the agarose to set.

The blocks were placed in a mixture of 1% Sarkosil (Sigma); 0,5 M EDTA pH 8; 2 mg/ml proteinase K (Merk) for 48 hrs. at 55° and subsequently rinsed with TE buffer three times. To inactivate proteinase K the blocks were then incubated with a 0,04 mg/ml PMSF solution for 1 hr at 55°C. The agarose plugs were digested with *SfiI* (Promega) for 15 hrs in the buffer recommended by the supplier. After extensive whashing with TE buffer the blocks were used for PFGE analysis.

4.3 CAT ASSAY

The vector used was pEMBL8CATO (kindly provided by Ciliberto and Cortese, EMBL, Heidelberg). HeLa cells were transfected with recombinant plasmids by the calcium phosphate precipitation technique (Graham (73)). 5 X 10^5 cells per dish were plated on the previous day in DMEM. 10 µg of high molecular wheight calf thymus DNA as carrier (Boehringer Manheim) were used for each transfection . 48 h after transfection, cells were harvested and extracts were prepared and heated at 60° C for 10 min. CAT assays were performed according to the method of Gorman (82). Reactions were carried out at 37° C for 15 min with 0,5 µCi of (^{14}C) chloramphenicol.

4.4 GEL RETARDATION ASSAY AND COMPETITION ANALYSIS

DNA fragments for gel retardation assay were isolated by electrophoresis on agarose gels and extracted using DEAE membranes. Oligonucleotide were purified from denaturing acrylamide gels, and annealed (Wu (87)). The concentration of purified fragments and oligonucleotides were determined by ethidium bromide staining (Maniatis (82)). Plasmid inserts and annealed oligonucleotide to be used as probes in band shift experiments, were end -labelled either with T4 polynucleotides kinase or Klenow fragment of *E. coli* DNA polymerase I as previously described (Falaschi (88)). Binding reactions were carried out by incubating 10^4 cpm of end labeled probe with 4 μg of HeLa cell nuclear extract prepared by the method of Dignam (Dignam (83)) and 3 μg of poly(d(I-C):poly(d(I-C) (Boehringer Manheim) in 20mM Hepes, pH 7.3, 50 mM NaCl, 4 mM $MgCl_2$, 2 mM DTT, 0,2 mM EDTA, 4 mM spermidine, 5% glycerol (final volume 20 μl). After 20 minutes incubation at room temperature, the protein-DNA complexes were resolved on a low-ionic strength 5% polyacrilamide gel.

Competition experiments were carried out by mixing a 10 to 40 fold molar excess of cold plasmid inserts or 20 to 180 fold molar excess of cold oligonucleotides to the probe before incubation with the nuclear extract.

DETECTION OF DNA-BINDING PROTEINS BY SOUTH-WESTERN ANALYSIS

South-Western experiments were performed by a modification of the method described by Silva (87). 200 to 600 µg of HeLa cell nuclear extract were submitted to SDS-PAGE using a 7.5% polyacrilamide Laemli gel. After electrophoresis, gel was soaked for one hour in 200 ml of renaturation buffer (10mM Tris-HCl, pH7.2, 50 mM NaCl, 20 mM EDTA, 1 mM DTT, 4 mM Urea), and then the proteins were electrophoretically transferred to nitrocellulose in 25 mM Tris base and 190 mM glycine. After transfer, the nitrocellulose strips were incubated for 1 hour in 100 ml Binding Buffer (10 mM Tris-HCl, pH 7.2, 50 mM NaCl, 5 mM $MgCl_2$, 0,1 mM EDTA, 1 mM DTT) containing 5% of non fat dry milk. After two washes in 100 ml each of Binding Buffer, the filters were incubated for one hour in Binding Buffer containing $5X10^5$ cpm per ml of ^{32P} -labeled oligonucleotide, and 20 µg per ml of non denatured sonicated salmon sperm DNA. Oligonucleotides were end-labeled either with T4 polynucleotide kinase or the Klenow fragment of *E coli* DNA polymerase I. The filters were then submitted to four subsequent washes in 100 ml of Binding Buffer each, for a total amount of time not exceeding 30 min. The strips were then dried for 15 min. and exposed overnight with an intensifying screen. All the binding and washing procedures were performed at room temperature.

4.6 RNA PREPARATION AND NORTHERN BLOT ANALYSIS

Total RNA was prepared using the guanidinium thiocyanate procedure (Maniatis). After gel electrophoresis (1% agarose) in 0,2 M formaldheyde (Lehrach (77)), the RNA (10 µg) was blotted to nitrocellulose membrane (Gene Screen) in 20 X

SSC and cross linked by UV treatment. Hybridization with (α^{32}P)dCTP-labeled probes (10^6 cpm/ml) prepared by nick translation (Rigby (77)) was performed in a solution of 45% (v/v) formamide, 0,2 M sodium phosphate (pH 7,2), 1 mM EDTA, 7% SDS, and 100 µg/ml denatured salmon sperm DNA at 45°C for 16 hr ((Joyner (85)). The membranes were subsequently washed in 40 mM sodium phosphate (pH7,2), 1% SDS, at 55 °C for 1 hr. (Church (84)).

4.7 NUCLEAR RUN-ON ANALYSIS

Nuclei were isolated by lysis of cells in a solution of 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 2 mM $MgCl_2$, and 0,1 % NP40 (modified from Groudine et al., 1981), resuspended at a concentration of $1X10^8$ to $2X10^8$ nuclei per ml in nuclear freezing buffer (40% (v/v) glycerol, 50 mM Tris-HCl (pH 8,3), 5 mM $MgCl_2$, and 0,1 mM EDTA), and stored at -80°c (Linial (85)). The nuclear run-on reaction was performed essentially as described by Linial et al. (85), incubating $2X10^7$ nuclei in a solution of 100 µl of 6 mM Tris-HCl (pH8), 2,5 mM $MgCl_2$, 150 mM KCl, 0,25 mM ATP 0,25 mM CTP, 0,25 mM GTP, 140 µCi of (α^{32}P)UTP (> 3000 Ci /mM, Amersham) and 60 U RNasin (Boehringer Manheim) for 20 min at 30°C. After purification of the labeled RNA (Linial (85)), hybridization to Gene Screen membranes containing 5 µg of the plasmid DNAs indicated in fig. 7 was done as described for Northern blot hybridization, except that *E. coli* RNA (250 µg/ml) was used as carrier.

4.8 TENIPOSIDE INCUBATION AND ISOLATION OF DNA

HL60 cells were washed twice with the culture medium RPMI (GIBCO) and resuspended at 6,6 X10^7 cells per ml. Teniposide (VM26, epipodophyllotoxin; Bristol Myers) was added at the concentration of 50 µM. After 30 min. at 37^0 C , the cells were pelleted , 70 µl of 20 mM Tris- HCl (pH 8) was added and mixed by inversion , and 3 µl of proteinase K was added. After overnight incubation at 37^0 c, the DNA was purified as described in Maniatis (82).

4.9 LIGATION MEDIATED PCR

For first strand synthesis 3 µg of HL60 Ava I digested chromosomal DNA and 0,3 pM of primer 1 were suspended in 15 µl of 40 mM Tris, pH 7,7, 50 mM NaCl. The sample was heated at 95^0 C for 2 min and then incubated at 60^{0C} for 30 min. Hybridization was stopped by transferring into ice; a solution of 7,5 µl of 20 mM $MgCl_2$, 20 mM DTT, and 0,02 mM of each dNTP was added, then 1 U of Sequenase (USB) was added and the sample incubated at 47^{0}C for 5 min. The reaction was stopped by heating at 60^{0}C for 5 min, then adding 6 µl of 310 mM Tris (pH 7,7), and then heating for 10 min at 67^{o}C. For ligation of linker the sample was transferred to ice, and a solution of 20 µl of 17,5 mM $MgCl_2$, 42,3 mM DTT, and BSA at 125 µg/ml was added, with 5 µl of PCR linker mix (20 pm of linker per µl in 250 mM Tris, pH7,7 and 3 Weiss units of T4 ligase per 25 µl) was added. After incubation over night at 15 ^{o} C the reaction was stopped by heating to 70^0 C for 10 min. The sample was precipitated, whashed with 70% ethanol and resuspended in water. 20 µl of 5X Taq buffer (200mM NaCl, 25 mM Tris pH 8,9, 25 mM $MgCl_2$, 0,05 % w/v gelatin) was added along with 20 nM of each dNTP, 10 pM of primer 2 and 10 pM of the longer oligomer of the linker, and 2,5 U of Taq polymerase (Cetus). The volume was

adjusted to 100 µl with water, heated at 95^0 C for 5 min, and then cycled in a PCR apparatus: 1 min at 95^0 C, 2 min at 63^{0}C and 3 min at 76^{o}C for 15 times. Samples were placed on ice , 1 to 5 pmol of an end labelled primer 3 was added along with 2,5 U of Taq polymerase and 20 nM of each dNTP. Samples were heated to 94^0 C for 2 min, hybridized at 66^0 C for 2 min, and extended at 76^0 C for 10 min. Polymerase activity was stopped by chilling on ice, adding 295 µl of 260 mM sodium acetate, 10 mM Tris pH 7,5, and 4 mM EDTA, and extracting with phenol-chloroform. The samples were precipitated, resuspended in water and one tenth of each sample was analyzed on a denaturing standard polyacrilamide gel.

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