ors12, a mammalian autonomously replicating DNA sequence, associates with the nuclear matrix in a cell cycle-dependent manner

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SUMMARY

Origin enriched sequences ors8 and ors12, have been isolated previously by extrusion of nascent CV-1 cell DNA from replication bubbles at the onset of S-phase. Both have been shown to direct autonomous DNA replication in vivo and in vitro. Here, we have examined the association of genomic ors8 and ors12 with the nuclear matrix in asynchronous and synchronized CV-1 cells. In asynchronously growing cells, ors8 was found to be randomly distributed, while ors12 was found to be enriched on the nuclear matrix. Using an in vitro binding assay, we determined that ors12 contains two attachment sites, each located in AT-rich domains. Surprisingly, in early and mid-S-phase cells, ors12 homologous sequences were recovered mainly from the DNA loops, while in late-S the majority had shifted to positions on the nuclear matrix. In contrast, the distribution of ors8 over the matrix and loop DNA fractions did not change during the cell cycle. By bromodeoxyuridine substitution of replicating DNA, followed by immunoprecipitation with anti-bromodeoxyuridine antibodies and PCR amplification, we demonstrated that ors12 replicates almost exclusively on the matrix in early and mid-S-phase; replicating ors8 was also found to be enriched on the matrix in early S-phase. Chase experiments showed that the ors12 sequences labelled with bromodeoxyuridine in the first 2 hours of S-phase remain attached to the nuclear matrix, resulting in an accumulation of ors12 on the nuclear matrix at the end of the S period.

Key words: ors12, mammalian autonomously replicating DNA sequence, nuclear matrix, cell cycle

INTRODUCTION

In the eukaryotic nuclei, chromosomal DNA is organized into topologically distinct loops anchored at specific sites (matrix attachment regions (MARs) or scaffold attachment regions (SARs)) to a proteinaceous framework, known as the nuclear matrix (nm) in nuclei, or chromosomal scaffold in metaphase chromosomes (Berezney and Coffey, 1974; Narayan et al., 1967; Zbarsky and Georgiev, 1959). These insoluble structures resemble morphologically the outline of the nuclei and chromosomes, respectively. They are prepared by extraction with high salt or detergent (LIS) of nuclease treated nuclei or chromosomes (Mirkovitch et al., 1984). There is accumulating evidence that the nuclear matrix plays an important role in the organization of many nuclear functions such as formation of DNA loops, DNA replication, transcription, higher order regulation of gene expression and chromatin assembly (Cook, 1991; von Kries et al., 1991; Pfeffer and Vidal, 1991; Jackson, 1990; Gasser and Laemmli, 1987; van der Velden and Wanka, 1987; Razin, 1987). This is also reflected in the association with the nuclear matrix of several enzymatic activities, such as primase, topoisomerase, RNA polymerase, DNA methylase and DNA polymerase (Cook, 1991; Razin, 1987; Tubo and Berezney, 1987; Collins and Chu, 1987; Wood and Collins, 1986; Berrios et al., 1985; Earnshaw et al., 1985); biologically active proteins, such as steroid receptors, p53, cyclin and c-myc (Nelson et al., 1986; Thompson et al., 1986; Celis and Celis, 1985; Reich and Levine, 1984); and other biological functions such as chromatin structure and assembly (Pfeffer and Vidal, 1991; Alique et al., 1990; Nelson et al., 1986; Earnshaw et al., 1985).

Microscopy studies have shown that nuclear DNA is replicated at a limited number of discrete sites corresponding to points on the nuclear matrix (for review see Jackson, 1990; Nakamura et al., 1986; Blow and Laskey, 1986), suggesting that DNA is replicated on replication complexes that are fixed on the nuclear matrix (Pardoll et al., 1980; Vogelstein et al., 1980; Dijkwel et al., 1979; Berezney and Coffey, 1975).

Studies showing preferential radiolabelling of the nuclear matrix-associated DNA (nnDNA) after short pulses have demonstrated that DNA replication occurs on the nuclear matrix. While the origins of DNA replication appear to remain fixed on the matrix (Cook, 1991; Dijkwel et al., 1986; Razin et al., 1986; van der Velden et al., 1984), DNA
sequences positioned away from initiation sites are eventually displaced into the DNA loops (Carri et al., 1986; Dijkwel et al., 1986; van der Velden et al., 1984; Pardoll et al., 1980; Vogelstein et al., 1980). Studies in yeast (Amati and Gasser, 1990; Brun et al., 1990) and in chicken erythroblasts (Razin et al., 1986) have shown that specific sites of initiation of DNA replication co-localize with the MARs. Recently, possible association of mammalian origins of replication with the nuclear matrix was further supported by the observation that a MAR is located in the initiation locus of the DHFR domain in CHO cells (Dijkwel and Hamlin, 1988).

We have previously generated a cloned library of early replicating nascent DNA extruded from small replication bubbles of CV1 cells synchronized at the G/S interface by aphidicolin and released briefly into S-phase (Zannis-Hadjopoulos et al., 1981; Kaufmann et al., 1985). Approximately 50% of the clones that we have examined, act as episomal origins of DNA replication in vivo, as tested by DpnI resistance, bromodeoxyuridine (BrDU) substitution and electron microscopic examination of replication bubbles formed on ors plasmids transfected into HeLa cells (Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991). We have recently demonstrated that these sequences can also function as plasmid origins of DNA replication in an in vitro replication system using HeLa cell extracts (Pearson et al., 1991).

In this study, two of the functional ors clones from this library, ors8 and ors12, were used to probe CV-1 genomic DNA fractionated into nmDNA and loop DNA under various experimental conditions. The data suggest that genomic ors12 associates with the nuclear matrix in a cell cycle-dependent manner, while genomic ors8 does not preferentially bind to the nuclear skeleton. In exponentially growing cells, there is a 3- to 6-fold enrichment of ors12 in the nmDNA by comparison to that found in the loops, while at G1/S transition or in early S-phase, ors12 is enriched 6- to 8-fold in the loop DNA fraction of CV-1 cells. The data also show that only the nuclear matrix-associated ors12 DNA is actively replicated in early S-phase and remains fixed on the nuclear matrix. Replicating ors8 is also enriched on the matrix in early S-phase.

MATERIALS AND METHODS

Ors plasmids

The cloning and characterization of ors8 and ors12 have been described previously (Kauffman et al., 1985; Zannis-Hadjopoulos et al., 1985; Frappier and Zannis-Hadjopoulos, 1987; Rao et al., 1990).

Synchronization of CV-1 cells

CV-1 cells were cultured in D5 (DMEM supplemented with L-glutamine, penicillin-streptomycin and anti-PPLO agent (Gibco) and 5% fetal bovine serum (FBS)) at 37°C. Cells were seeded at 2×10⁶ to 3×10⁶ per 150-mm plate and were 30-40% confluent after growing for 14-18 h at 37°C. For the experiments requiring synchronization, cells were treated as described previously (Kauffman et al., 1985; Ward et al., 1991). Briefly, each plate of cells was washed twice with DV0 (supplemented DMEM with no serum) and incubated in 16 ml of the same media for 24 h. Next, the cells were synchronized at the G1/S boundary by the addition of FBS and aphidicolin (Sigma) to a final concentration of 20% (v/v) and 2 µg/ml, respectively. After 18 h at 37°C the cells were either harvested or released into S-phase by two rinses of DVO and reincubation in DV10 (supplemented DMEM with 10% FBS) for designated time points.

Flow cytometric analysis of DNA content

The DNA content and synchrony of all cells were monitored by flow cytometry using a FACS III instrument equipped with a 5 W argon ion laser adjusted to emit at 488 nm. Prior to preparation of nuclear matrices, aliquots of 2×10⁶ cells were removed for flow cytometric analysis. Cells were harvested by centrifugation, washed twice with PBS and resuspended in 50 µg/ml propidium iodide (PI), 10 mM Tris-HCl, pH 7.0, 5 mM MgCl₂ to a final cell concentration of 2×10⁶ cells/ml. RNase was added to a final concentration of 30 µg/ml and the PI-cell suspension was stored at 4°C until flow cytometric analysis could be performed.

Preparation of nuclear matrix DNA fractions

Preparations of nmDNA were made using the high salt extraction method (Hodge et al., 1977) with the following modifications. The typical preparation began with a pellet of 2-4×10⁸ cells (20×150 mm Petri plates at 60-70% confluence). Cells were resuspended in 100 ml of BSA-PBS (2% bovine serum albumin in phosphate-buffered saline containing 0.1 mM PMSF), pooled into two or three 50 ml conical centrifuge tubes and collected by centrifugation at 900-1100 g. In each tube the cells were washed once with 50 ml of BSA/PBS and once with 50 ml of PBS containing PMSF only. Each tube of cells was resuspended in 40 ml of 1× RSB hypotonic buffer (10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 10 mM NaCl, 0.1 mM PMSF) containing 0.5 mM CuSO₄ for stabilization of the nuclei and placed on ice for 10 min. After centrifugation, the cells were resuspended in a total volume of 10 ml of 1× RSB buffer containing 0.3% NP40 and homogenized with twenty strokes of a Dounce homogenizer (paddle B). The homogenate containing nuclei was then dispensed as 1 ml samples into ten to fifteen 1.5 ml microfuge tubes. The nuclei were collected by centrifugation at 300 to 400 g for 3-5 min in a Beckman 12 microfuge at 4°C and subsequently washed 2-3 times with the homogenization buffer (1 ml per microfuge tube). Nuclei were either resuspended in 50% glycerol/homogenization buffer and stored at −70°C or immediately prepared for digestion with EcoRI.

The nuclei in each tube were washed 4 times with 1 ml of buffer (10 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 0.03% NP40, 0.1 mM PMSF), resuspended in 200 µl of 1× react 3 buffer (Gibco-BRL) and digested with 150 units of EcoRI overnight at 37°C. An equal volume of 50 mM Tris-HCl, pH 8.0, 4 M NaCl was then added and the mixture was allowed to sit at room temperature for 10-20 min. Nuclear matrix material was pelleted by centrifugation in the microfuge for 2 s at maximum force and the supernatant was collected and digested with proteinase K (1-2 mg/ml final concentration). The nm material was redigested and re-extracted with high salt twice more, as above. The final nuclear pellet was resuspended in 200 µl of Hirt’s lysis buffer (50 mM Tris-HCl, pH 8.0, 0.6 M NaCl, 1 mM EDTA, 0.5% SDS) and digested with 3 mg/ml proteinase K at 45°C overnight. The high salt extracts and proteinase K-solubilized nuclear material were extracted twice with phenol:chloroform (4:1) and twice with chloroform. The pooled high salt extracts comprise the loop DNA fraction while the proteinase K-solubilized nuclear material comprise the nmDNA fraction.

Preparation of DNA blots for hybridization

Samples (20 µg each) of DNA from the DNA loop and nmDNA fractions were digested to completion with HindIII and PstI and
subjected to electrophoresis in 0.7% agarose gels (TBE buffer system). DNA in the gel was transferred under vacuum onto Zetaprobe (Bio-Rad) or GeneScreen Plus™ (New England Nuclear) membrane using a LKB 2016 VacuGene apparatus (Pharmacia-LKB) and protocols recommended by the manufacturers. [35S]dCTP radiolabelled DNA probes were prepared using the Pharmacia Oligolabelling kit (Pharmacia-LKB) and hybridization was carried out at 65°C as described previously (Zannis-Hadjopoulos et al., 1985).

**In vitro binding**

Matrix-halo structures were prepared from CHO cells by the LIS extraction procedure (Mirkovitch et al., 1984) as previously described (Dijkwel et al., 1991). The matrices were suspended in 50 mM NaCl, 10 mM MgCl₂, 50 mM Tris-HCl, pH 7.4, and 250 i.u./ml DNase I (BRL) was added. Digestion was allowed to proceed for 45 min at 37°C, after which EDTA was added to 50 mM. The matrices were then collected by centrifugation (10 min at 4000 r.p.m. in a Sorvall HB4 rotor) and were washed three times with binding buffer (BB: 20 mM KCl; 70 mM NaCl; 20 mM EDTA; 20 mM Tris- HCl, pH 7.4).

ors-containing plasmids, digested with the appropriate restriction enzyme(s), were end-labeled by filling in of staggered ends with Klenow and the appropriate [α-32P]dNTP.

To detect affinity of ors sequences for the nuclear matrix, samples of the matrix suspension (equivalent to 1-2×10⁶ cells) were incubated for 2 h at 37°C in 400 µl of BB with 20 ng of end-labeled fragments (~1.5×10⁵ d.p.m.) in the presence of increasing amounts of non-specific and specific competitor DNA. pBR322 digested with HindIII was used as the non-specific competitor, while a HindIII digest of a plasmid (pMAT3.4) containing the MAR located in the initiation locus of the DHFR domain in CHO cells (Dijkwel and Hamlin, 1988), was chosen as the specific competitor. The incubation was terminated by adding 600 µl of ice-cold BB and spinning down the matrices in a Beckman microfuge. The matrices were washed once with 1 ml of BB and were then dissolved in 0.5% SDS, 20 mM EDTA, 10 mM Tris-HCl, pH 7.4. Proteinase K was added to 1 mg/ml and after a 3-5 h incubation at 37°C, the aliquots were directly loaded into the wells of a 1% agarose gel. After electrophoretic separation, the DNA was transferred to a membrane and autoradiography was performed using Kodak X-Omat AR film.

**Preparation of BrdU-labelled DNA**

CV-1 cells blocked at the G1/S interface by aphidicolin treatment were released by washing twice in PBS and pulse-labelled in DV10 containing 100 µM BrdU for 5 min. The cells were then washed twice in PBS and incubated for 1-3 h with complete DV10 to chase the BrdU label. NmDNA fractions were prepared by digesting with EcoRI, as described above. The purified DNA fractions were digested with HindIII and PstI.

**Immunoprecipitation of BrdU-labelled DNA**

Equal quantities (0.25 µg in 100 µl of 10 mM Tris-HCl, 1 mM EDTA, pH 8) of digested BrdU-labelled mmDNA and loop DNA, were denatured by boiling for 5 min and incubated with 1 µl of 100 ng/µl monoclonal antibody to BrdU (Boehringer Mannheim), on ice for 1-2 h. Antibody-bound DNA was extracted from unbound DNA using rabbit anti-mouse IgG conjugated to polyacrylamide beads (Immunobeads; Bio-Rad), as described previously (Bell et al., 1991). Briefly, 245 µl of prewashed immunobeads were added to the mixture and incubated on ice for 1-2 h with occasional mixing. The DNA-antibody-immunobead complexes were collected by centrifugation and the pellet was resuspended and washed three times in 1 ml each of (a) 0.1% BSA, 0.5 M NaCl, 10 mM Tris-HCl, pH 7.4 and (b) 0.1% NP40, 0.15 M NaCl, 10 mM Tris-HCl, pH 7.4. BrdU-labelled DNA was eluted from the immunobead pellet by the addition of 200 µl of TE buffer (pH 7.4) containing 2% SDS. Removal of the immunobeads and extraction of the DNA was done as described previously (Bell et al., 1991). The immunoprecipitated DNA was collected by ethanol precipitation and used as template for PCR amplification.

**PCR amplification of immunoprecipitated BrdU-labelled DNA**

The BrdU-labelled DNA was resuspended in 69 µl of sterile water and the following were added to make up the final 100 µl reaction volume: 1 µl of 400 ng/µl primer of ors12 (sequence of nucleotide position 187 to 202 of top strand, complementary to bottom strand of ors12 sequence), 1 µl of 400 ng/µl of – primer of ors12 (sequence of nucleotide position 768 to 783 of top strand, complementary to top strand), 10 µl of 10× PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 1 mg/ml BSA), 3 µl of 50 mM MgCl₂ and 16 µl of nucleotides (200 µM dNTP). The reaction was carried out in a thermal cycler (Perkin Elmer Cetus) by the addition of 1.25 units of Taq polymerase (Pharmacia) per 100 µl reaction using the following thermocycling program: 1 cycle of 94°C (1 min); 30 cycles of (94°C (1 min); 37°C (1 min); 72°C (1 min)); and 1 cycle of 72°C (5 min). Then 25 µl of amplified products from each 100 µl PCR reaction were separated on a 1% agarose gel (TBE buffer system), Southern blotted and hybridized with 32P-labelled DNA fragments produced from the 215 nucleotide Scal-Styl fragment of ors12.

**Quantitation of ors sequences**

ors sequences were quantitated by densitometry scans of the hybridization signals on autoradiographs using Bioimage (Milligen/Bioresearch, Division of Millipore).

**RESULTS**

**Description ors8 and ors12 DNA sequences**

The various topological features of ors8 and ors12 are summarized in Fig. 1 (Rao et al., 1990). Restriction mapping of CV-1 genomic DNA revealed that ors8 (484 nucleotides in length) is contained within a 1.2 kb PstI fragment (unpublished results), and ors12 (812 nucleotides in length) resides in a 1.4 kb HindIII fragment (Mah et al., 1992). A portion of the non-α-satellite region of ors12 (215 bp long Scal-Styl fragment; <9 copies per haploid genome; Mah et al., 1992) was used as probe for the identification of ors12-containing genomic DNA sequences, while for identification of ors8-containing genomic DNA the entire ors8 sequence (<5 copies per haploid genome; Zannis-Hadjopoulos et al., 1985) was used.

**Determination of non-specific binding to nuclear matrix**

Since preparations of nuclear matrix can bind DNA non-specifically (unpublished observations), we determined the association of exogenous radiolabelled DNA with the nuclear matrix occurring during our matrix isolation procedure. Lambda DNA, digested with HindIII and labeled with [α-32P]dCTP by Klenow repair of staggered ends (Maniatis et al., 1982), was added to the nuclear pellet at a 250-fold excess (compared to the amount of ors12 relative to CV-1 genomic DNA) and its adherence to the
nuclear matrix was determined. In three separate experiments, we found that only a negligible amount (average of 0.78%) of the $\lambda$ HindIII DNA bound to the nuclear matrix after the first cycle of EcoRI digestion/high salt extraction, and after the third cycle this value had dropped to an average of 0.41% (Table 1). In contrast, the amounts of genomic CV-1 DNA still attached to the matrix were 68% and 7.5% of total DNA, respectively, after the first and third extraction cycles.

**Predicted ORS distribution on the nuclear matrix**

Our method of preparation of nuclear matrices typically yielded approximately 10% of the total DNA being associated with the nuclear matrix. The expected distribution of ORS sequences over the nm and loop-DNA fractions, is summarized in Fig. 2. Binding to the nuclear matrix is considered to be at or below background levels when the hybridization signal of the nmDNA is 1/28 or less of the hybridization signal of the loop DNA (see Table 1).

**ors12, but not ors8, is enriched on the nuclear matrix of exponentially growing CV-1 cells**

To determine the position of genomic ors8 and ors12 relative to the nuclear matrix, nuclei isolated from exponentially growing CV-1 cells were extracted with high salt and digested with EcoRI. The sample was then divided in two: one half was digested with PstI and the other with HindIII. The entire matrix-attached (nm) and loop DNA fractions were isolated, separated on agarose gels and transferred to filters. Fig. 3A shows that when the filter was hybridized with a probe specific for ors12, the signals in the nm and loop lanes were of approximately similar intensity. However, as nmDNA constitutes only 10% of the total DNA complement, this result indicates an approximately 5-fold enrichment of ors12 in the matrix fraction. In contrast, the majority of ors8 (Fig. 3B) was recovered in the loop fraction.

To corroborate this observation, the above experiment was repeated in a slightly different way. Instead of equal cell equivalents, equal amounts (in $\mu$g) of the loop and nm DNA were separated on agarose gels. Fig. 4A shows that in all three preparations of log-phase CV-1 cells analyzed, ors12 was enriched in the nmDNA fraction. Densitometric analysis of the hybridization bands indicates, on average, a 4.3-fold enrichment of ors12 in the matrix fraction. In contrast, the majority of ors8 (Fig. 3B) was recovered in the loop fraction.

Table 1. Background binding of $^{32}$P-labelled lambda HindIII DNA to nuclear matrix of log CV-1 cells

<table>
<thead>
<tr>
<th>Extraction</th>
<th>% Binding of labelled lambda HindIII DNA*</th>
<th>% Of DNA retained in nuclear matrix pellet†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st Extraction</td>
<td>0.78</td>
<td>68</td>
</tr>
<tr>
<td>2nd Extraction</td>
<td>0.53</td>
<td>33</td>
</tr>
<tr>
<td>3rd Extraction</td>
<td>0.41</td>
<td>7.5</td>
</tr>
</tbody>
</table>

*Nucleoid pellet incubated with $2.24 \times 10^6$ c.p.m./0.5 mg of $^{32}$P-labelled lambda DNA.
†Total DNA including all extractions and nuclear matrix fraction = 147 $\mu$g.

Fig. 2. Predicted distribution of ORS sequences on nmDNA and loop DNA fractions. The index: 
\[
\frac{\text{proportion of total DNA (µg)}}{\text{proportion of ORS copies}}
\]

was used to calculate the ratio of hybridization band intensities of loop DNA to nmDNA on autoradiographs ($X_{\text{loop DNA}}:X_{\text{nm DNA}}$) when equal amounts of DNA are analyzed (10 µg/lane). The predicted appearance of Southern blot results was determined using this ratio.
ors12 associates with the nuclear matrix

**Fig. 3.** *ors8* and *ors12* associate with the nuclear matrix to different extents. (A) Nuclear matrices were isolated from exponentially growing CV-1 cells. After digestion with *Eco*RI and *Hind*III, the entire nm- and loop-DNA fractions were isolated, separated on an agarose gel, transferred to a membrane and hybridized to a low-copy probe specific for *ors12* (215 bp *Scal/Sty* fragment). Left set of 3 lanes contain DNA prepared from nuclear matrix pellets (nmDNA) and high salt extracts (loop DNA) that was either re-digested with *Hind*III (+ lanes) or not (− lane). Right set of 2 lanes indicate the position of the bands generated by complete digestion of CV-1 DNA with *Hind*III (1.4 kb) and *Pst*I (3.1 kb). (B) As (A) except that CV-1 DNA was digested with *Pst*I and the blotted membrane was hybridized to a probe specific for *ors8*.

**Fig. 4.** *ors12* is preferentially associated with the nuclear matrix of asynchronous CV-1 cells, whereas *ors8* is not. Nuclear matrices were prepared from exponentially growing CV-1 cells and digested with either *Eco*RI and *Hind*III or *Eco*RI and *Pst*I. Equal amounts (20 µg) of nm and loop DNA were separated on 0.8% agarose gels and transferred to a membrane. The membranes were probed for (A) *ors12* and (B) *ors8*, as in Fig. 3. The relative hybridization signals of loop DNA and nmDNA fractions are shown for three different batches of log CV-1 cells (numbered 1 to 3). Quantitation of the hybridization signals using BioImage yielded ratios of (loop *ors12*:nm *ors12*) of 1:3.0, 1:3.4 and 1:6.0 for batches 1, 2 and 3, respectively. For *ors8* the corresponding values were 1:1.3, 1:1.2 and 1:0.79, respectively.
ors8. In contrast to ors12, ors8 is approximately equally distributed over the nm and loop DNA fractions (Fig. 4B), as would be expected for a randomly distributed sequence (viz. Fig. 2).

**Attachment of ors12 to the matrix is mediated by the AT-rich domains**

ors8 is almost uniformly AT-rich, whereas ors12 consists of three distinct domains: a 178 bp stretch of AT-rich α-satellite DNA at the 5'-end, which is separated from a non-satellite AT-rich region at the 3'-end by a 450 bp region containing three IRs and the β-globin upstream transcriptional control sequence (CACCC) (Fig. 1; Rao et al., 1990). We used the assay developed by Cockerill and Garrard (1986) to determine whether ors8 and ors12 are capable of binding to the matrix in vitro. Plasmids containing ors8 were digested with Hinfl, while those containing ors12 were digested with XmalIII, ScaI, StyI and BglII (Rao et al., 1990). The fragments were labeled by filling in the staggered ends with Klenow polymerase using 32P-labelled nucleotides. The results (Fig. 5A) show that in the absence of competitor DNA all fragments exhibit affinity for the nuclear matrix. However, when pBR322 DNA is present as competitor, only a 400 bp fragment (*) binds to the matrix. As both the α-satellite and the 3' AT-rich segment reside in fragments of this size, the experiment was repeated using a BglII/Styl digest of the ors12 plasmid, allowing discrimination between the two AT-rich domains. Both the 620 bp fragment (*) containing the satellite sequences (5'-end portion) and the 425 bp fragment (**) harboring the 3' AT-rich DNA were found to bind (Fig. 5B). Thus at least two distinct sites of contact exist between the matrix and ors12. Though the nature of the binding is not understood, our observation that ors8 does not show a similar affinity for

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**Fig. 5.** Only ors12 binds to the nuclear matrix in vitro. Nuclear matrices, prepared from exponentially growing cells, were treated with DNase I to remove all genomic DNA. To aliquots of the matrix preparation, end-labelled restriction fragments obtained from ors-carrying plasmids were added. After 2 h at 37°C, matrices were collected by centrifugation and bound DNA was purified. After separation on 1.5% agarose gels, the DNA was transferred to a filter, which was exposed to film. The matrix-bound fragments are indicated (*, **). (A) BglII/XmalIII/ScaI/Styl digest of ors12; (B) BglII/ScaI/Styl digest of ors12; (C) Hinfl digest of ors8; (D) Hinfl digest of pMAT3.4 (a plasmid containing the MAR located in the initiation locus of the DHFR domain in CHO cells). Size markers: M1, 1 kb ladder; M2, 123 bp ladder. Competitors: specific, pMAT3.4 (pMAT); non-specific, pBR322 (pBR); see Materials and Methods for experimental details.
ors12 associates with the nuclear matrix

The matrix (Fig. 5C) suggests that a high AT-content by itself is probably not sufficient for matrix-binding.

Binding of the ors12 fragments to the matrix is not abolished by substituting the pBR322 DNA with the specific competitor pMAT3.4, a plasmid containing the previously characterized MAR detected in the initiation locus of the DHFR domain in CHO cells (Dijkwel and Hamlin, 1988) (Fig. 5A,B). This suggests either that ors12 and the DHFR MAR bind to the matrix at different sites or that ors12 has a significantly higher affinity than the DHFR MAR for the same site. As a positive control, Fig. 5D shows that a 480 bp HindIII fragment (+) is responsible for specific binding of the DHFR MAR to the matrix. As expected, this binding is abolished when excess unlabeled pMAT is present in the binding assay.

**ors12 accumulates on the nuclear matrix in cells progressing through S-phase**

To determine the distribution of genomic ors sequences over the nm and loop-DNA fractions as a function of position in the cell cycle, CV-1 cells were arrested in G0 by serum starvation and were then released into complete medium containing aphidicolin for 12 hours. Surprisingly, in cells collected at the G1/S interphase, ors12 is found predominantly in the loop fraction. When cells, released from the aphidicolin block into complete medium for 2 hours are analyzed, this distribution is essentially similar. However, at 6 hours into S, ors12 is observed to be slightly enriched in the matrix as compared to the loop DNA fraction, and its distribution starts to resemble that observed in log-phase cells (Fig. 6A).

In contrast, ors8 is again found to be distributed essentially randomly over the nm and loop DNA fractions and this distribution, which is similar to that found in log-phase cells, remains essentially unchanged through the S period (Fig. 6B).

**Localization of actively replicating genomic ors8 and ors12 sequences**

To investigate whether the nm is the site of genomic ors replication, we pulse-labeled CV-1 cells with [3H]thymidine for periods of 5, 30, 60 and 120 minutes, starting immediately after their release from the aphidicolin block, and measured the amount of radioactivity in the nm- and loop DNA fractions (Fig. 7). As expected, overall specific activity of both the nmDNA and loop DNA increases with the increasing labelling time. However, after a 5-min pulse the specific activity of nmDNA is approximately 5-fold higher than that of loop DNA. This value decreases to approximately 1.3-fold after a 30 or 60 minute pulse, while after a 2-hour pulse it is comparable to that of loop DNA. These data suggest that the nuclear matrix is the site of DNA replication, as has been suggested before by others (Vogelstein et al., 1980). The increase in the specific activity of loop DNA with increasing pulse times relative to that of nmDNA suggests that some of the DNA associated with the nuclear matrix at the time of the pulse moves out into the DNA loops as time progresses.

In view of the fact that the association of genomic ors12 with the nuclear matrix varies with the cell cycle, and in order to determine whether localization of ors12 on the matrix versus the loop is important for its being actively replicated, synchronized CV-1 cells were pulse-labelled with BrdU for 2 minutes immediately after release from the aphidicolin block, and at several time points (up to 1.5 hours) thereafter. Cell synchrony was monitored by flow...
cytometry (Fig. 8D), as previously (Kaufmann et al., 1985). Equal quantities of HindIII-digested DNA from the nm and loop DNA fractions were then immunoprecipitated using mAb to BrdU and the ors12 content of the immunoprecipitated DNA was estimated by southern blot hybridization after PCR-amplification of the ors12 DNA (596 bp fragment, Fig. 8A; ors12 products generated by PCR sometimes appear as a doublet, which is also present in the control, when the ors12 plasmid is used as template (right-most lane Fig. 8B)). We invariably observed that, immediately after the pulse, the BrdU-substituted genomic ors12 sequences were positioned predominantly, and often exclusively, on the matrix (Fig. 8A). This contrasts to the bulk of the ors12 sequences, which in early S-phase, are predominantly present in positions in the DNA loops (Fig. 6A).

Interestingly, when the BrdU pulse, administered immediately after release of cells from the aphidicolin block, was followed by a 1.5 hour chase in regular medium, the position of ors12 sequences did not appear to change. Only after a 3 hour chase were we able to detect a relatively faint ors12 signal in the loop fraction (Fig. 8A). These results strongly suggest that genomic ors12 sequences replicate on the nuclear matrix and that, once replicated (at least those in early S), remain attached.

The same analysis was done for BrdU-substituted ors8 sequences. Equal quantities of PstI-digested DNA from the nm and loop DNA fractions were immunoprecipitated with anti-BrdU mAb and the ors8 content of the immunoprecipitated DNA was estimated by Southern blot hybridization after PCR-amplification of the ors8 DNA (495 bp fragment, Fig. 8C). In contrast to ors12, at all the time points examined, labeled ors8 was found on both the loop and nm DNA fractions. By densitometric analysis of the bands in Fig. 8C it was estimated that the ratio of BrdU-labeled DNA in the loop versus the nuclear matrix remained unchanged up to 3 hours into S-phase and was approximately 2:1. These results indicate no significant change from the overall distribution profiles of ors8 sequences during the cell cycle (Fig. 6B).

Finally, we analyzed whether the distribution of newly replicated ors8 differed from that of ors12. Synchronized cells were pulse-labeled with BrdU for 10 minutes and 30 minutes immediately after release from the aphidicolin block. Replicated DNA was then immunoprecipitated and analyzed on Southern blots after PCR amplification of the ors8 and ors12 sequences. The results (Fig. 9) show that again ors8 and ors12 behave differently. Though after a 10-min pulse these differences are somewhat masked by the contribution of attachment of the replication forks to the matrix, after a 30-min pulse ors12 is found exclusively in the matrix fraction (Fig. 9A); ors8, on the other hand, although found predominantly in the matrix fraction, it is also detected in the loops (Fig. 9B), suggesting that at least some of the early replicating ors8 homologous sequences are replicated passively. Interestingly, the BrdU-substituted ors8 was positioned predominantly on the nuclear matrix at both 10 and 30 minutes into S (Fig. 9B), in contrast to the finding that at all stages of S, and indeed at all times of the cell cycle examined, ors8 sequences are nearly equally distributed on the loop and nm DNA fractions (Fig. 6B).

**DISCUSSION**

Conflicting views exist regarding the position of origins of DNA replication relative to the nuclear matrix. Some authors propose that origins are permanently associated with the matrix (van der Velden et al., 1984; Dijkwel et al., 1986; Razin et al., 1986; Dijkwel and Hamlin, 1988), since label administered in early, but not in late S-phase, clearly remains in close proximity to the nuclear matrix. Others support the notion that origins of DNA replication are temporarily associated with the matrix to facilitate initiation of DNA replication, but later move out into the DNA loops (Cook and Lang, 1984). Evidence for this model is indirect: mammalian sequences, which are capable of directing autonomous DNA replication in yeast, are found both on the nm and in the DNA loops. As these sequences are assumed to be replication origins and as replication occurs on the nuclear matrix, it is assumed that the ARS-elements move from one position to the other.

In recent years, we have isolated and characterized sequences from mammalian cells implicated in initiation of DNA replication. These ors elements were obtained by extrusion of short nascent strands from DNA isolated from cells immediately after entry into the S-period (Kaufmann et al., 1985). When present on plasmids, most of the ors sequences analyzed have been shown to direct autonomous replication both when transfected into animal cells (Frapier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991) and in a recently developed in vitro replication system (Pearson et al., 1991). These data further support the idea that ors sequences may actually be origins of replication in their chromosomal context, although this is not yet directly proven. Consequently, we felt that we could address the question whether mammalian origins are permanently fixed on the nuclear matrix or...
ors12 associates with the nuclear matrix

whether their association is of a more dynamic nature. Our study focussed on two of the best characterized ors, ors8 and ors12.

Analysis of matrix-attached and loop-DNA isolated from exponentially growing CV1 cells, showed that ors12 is enriched in the matrix fraction while ors8 exhibits an essentially random distribution (Fig. 4). This observation was confirmed in an in vitro binding assay, which showed that ors12 but not ors8 has affinity for the nuclear matrix (Fig. 5). This is somewhat surprising as both ors8 and ors12: (1) were isolated and cloned in the same manner (Kaufmann et al., 1985); (2) share similar sequence characteristics (Rao et al., 1990); and (3) have been shown to act as episomal origins of replication in vivo (Frappier and Zannis-Hadjopoulos, 1987) and in vitro (Pearson et al., 1991). The major difference between them is the presence of α-satellite sequences in ors12 and their absence in ors8. Furthermore, ors12 was recently localized, through its non-α-satellite portion, at the centromeric region of six CV-1 chromosomes (B4, C11, D14, D24, E25, E27) and a marker chromosome (Mah et al., 1992). It is, therefore, possible that ors8 and ors12 represent different classes of cellular replication origins.

Our data also show that association of genomic ors12 with the nuclear matrix is cell cycle-dependent: in early S, ors12 sequences were barely detectable in nmDNA, while at 6 hours into S an enrichment was seen in this fraction, as was also seen in exponentially growing cells (Fig. 6A).
This cell cycle-dependency may be reflective of binding of α-satellite DNA to the nuclear matrix in a process related to the assembly of the replicated chromosomal centromeres (Schulman and Bloom, 1991). This notion is supported by the recent description of a nuclear matrix protein that binds specifically to α-satellite DNA (Poltoratskii et al., 1991).

The accumulation of replicated ors12 on the matrix could be followed by BrdU-labelling. When CV-1 cells, synchronized at the G1/S interface with aphidicolin, were released into the S period and were then pulse-labelled with BrdU either immediately or after 90 minutes, labeled ors12 was exclusively found on the matrix, irrespective of the length of the pulse. This distribution essentially did not change when the pulse was followed by a chase of up to 3 hours (Fig. 8A). In contrast, similar analysis of BrdU-substituted ors8 showed that at all the time points examined, labeled ors8 was found on both the loop and nm DNA fractions at a ratio of approximately 2:1 (Fig. 8B). Therefore, while early replicating ors12 exhibits binding documented before as being characteristic of replication origins (Carri et al., 1986; Dijkwel et al., 1986), some of the ors8 related sequences do not appear to associate with the matrix in the same way. Our results, therefore, suggest that only a subset of early replicating ors8 and all of early replicating ors12 might actually be recruited as origins at the onset of S-phase. This observation might be explained by sequential recruitment of specific DNA sequences to act as origins of replication during the course of the S period. Our previous studies have shown that ors8 is replicated at 1-6 hours into the S-phase (Zannis-Hadjopoulos et al., 1988).

The above observations are not unlike the situation with yeast ARS elements, of which only a subpopulation is recruited as active replication origins in the chromosome (Dubey et al., 1991). Possibly, localization with respect to the nuclear matrix is the deciding factor.

In CV-1 cells there are no more than nine copies of ors12 all of which are located at the centromeric regions of six different chromosomes and a marker chromosome (Mah et al., 1992). It is, therefore, conceivable that only ors12 sequences located in a particular chromosome or subset of chromosomes function as replication origins in early S-phase. Alternatively, all ors12 sequences can potentially function as replication origins even though they are located on different chromosomes, but only a sub-population of each is actually being recruited to be active in early S.

MARs and SARs, which anchor DNA to the nuclear matrix and the bases of the DNA loops, are typically found at the boundaries of functional transcriptional units (von Kries et al., 1991; Phi-van and Stratling, 1990; Gasser and Laemmli, 1987; Gasser and Laemmli, 1986b) and there is abundant evidence suggesting that MARs/SARs play an important role in transcriptional control (Phi-van and Stratling, 1990; Phi-van et al., 1990; Stief et al., 1989; Gasser and Laemmli, 1986b; Ciejak et al., 1983). Topoisomerase II has been described as a major nuclear matrix protein (Berrios et al., 1985) and it has been shown to bind MARs/SARs in vitro (Adachi et al., 1989). It is conceivable, however, that other proteins in the nuclear matrix have a similar capacity (von Kries et al., 1991). As it associates with the nuclear matrix in a non-random manner, ors12 probably harbors one or possibly two (Fig. 5) sites that can interact with the DNA-binding matrix proteins. The observed sequential activation of ors12 could then be explained by accumulation of an origin-specific protein during the S period, which binds to ors12 allowing it to fire. Alternatively, an existing protein could be modified to act similarly. This model clearly predicts that all ors12 sequences would act as replication initiation sites when in their normal chromosomal context. However, our present data do not exclude the possibility that only the fraction of ors12 that is replicated in early S, be an actual origin; the remainder may be replicated passively from adjacent origins and may accumulate on the matrix in the course of S-phase because of its location near a termination site. We are currently addressing these issues by two-dimensional replicon mapping approaches.

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