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The spatial organization of sequences involved in initiation and termination of eukaryotic DNA replication

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ABSTRACT

Nuclear DNA is looped by attachment to a matrix or cage. As this cage is the site of DNA synthesis, sequences in the loops must attach before they are replicated. We have tested whether sequences which initiate replication are usually out in the loop and attach only during S phase or whether they are attached but quiescent during most of the cell-cycle. Sequences which permit plasmids to replicate autonomously in yeast cells (ARS's) are strong candidates for initiating sequences. Four different human ARS's all map remote from attachment points to the HeLa nuclear cage. In addition a potential terminus of replication is also remote from the cage. We conclude that sequences involved in initiation are usually out in the loop and that DNA synthesis is initiated by their attachment.

INTRODUCTION

Recent evidence shows that linear nuclear DNA is organized into a series of loops by attachment to a larger sub-nuclear structure, called variously the nuclear matrix, scaffold or cage. (See refs. 1, 2, for reviews). This sub-structure is also the site of DNA synthesis (3-5), so that DNA in the loops must attach before it is replicated. Sequences which initiate replication (i.e. origins) might usually be out in the loop, attaching only during S phase: alternatively, they might be attached but quiescent during most of the cell-cycle, awaiting activation during S phase. We wished to establish which of these alternatives is most likely.

If origins are generally attached, even in the absence of DNA synthesis, they should be enriched in the fraction of total DNA that resists detachment by a nuclease from the sub-structure. The sites involved in initiating replication are poorly characterized: for example, there remains no direct proof that replication even initiates at specific DNA sequences (6). Strong evidence, albeit indirect, for

specificity is provided by the isolation of specific sequences (ARS's) that permit plasmids to replicate autonomously in yeast (7): presumably ARS's - which have now been isolated from various eukaryotes (8, 9) - provide an initiation site for yeast plasmids that lack one. Therefore, as ARS's are strong candidates for initiating sequences, we compared their relative concentrations in total DNA and the DNA that resisted detachment. We also mapped the positions of four human ARS's relative to attachment points and found that all were located out in the loops. In addition we mapped a sequence that lies immediately adjacent to a candidate for a terminus of replication in the rat genome (10). This chromosomal region defines one limit of the local endoreduplication induced by mitomycin C of a viral sequence integrated in the rat chromosome. It, too, lies far from an attachment point.

MATERIALS AND METHODS

Isolation of human ARS's

The 4 human ARS's were isolated as described in the accompanying paper (9) and their properties are summarised in Table 1. One was derived from total placental DNA, two from total HeLa DNA and one from HeLa DNA that remained closely associated with nucleoid cages after detaching all but 0.8% of the total with Eco R1 (11, 12).

Detachment mapping

The positions of the human ARS's were mapped relative to attachment points using HeLa nucleoids (11, 12). The position of a 0.9 kb rat Eco R1 fragment carried in plasmid pSCL1 (10, 13) was mapped similarly using nucleoids from 82 or VIT cells (12). [These are rat cells transformed by polyoma and avian sarcoma virus respectively]. This 0.9 kb fragment is immediately adjacent to a candidate for a terminus of replication in the rat genome.

RESULTS

Two kinds of DNA were prepared from nucleoids isolated by lysing unsynchronized HeLa cells in a non-ionic detergent and 2M NaCl. [Unsynchronized cells can be used for these experiments since <1% of the loops are replicating at any time (5).]. Some nucleoids, which contain naked and looped DNA packaged within a protein cage, provided a source of total DNA; the rest were incubated with Eco R1 to detach from the cage all but 0.8% of the total. Both samples were purified, completely redigested

Table 1. Detachment mapping 4 human ARS's and one prospective rat terminus of replication in various nucleoids

PLASMID	SOURCE OF INSERT DNA	DETACHMENT MAPPING			
		Nucleoid	Size of fragments hybridizing (kb)	%DNA remaining	Relative enrichment
ARS probes					
pMA51	Total placental	HeLa	8.2, 1.8, 1.5	6%	1.0 x
pMA52	Total HeLa	HeLa	2.5	4%	0.7 x
pMA53	Cage HeLa (0.8% remaining)	HeLa	6.7	4%(a)	0.8 x
pMA54	Total Hela	HeLa	9.6, 4.0	4%	0.8 x
Terminus probe					
pSCL1	Total Rat	VIT	0.9	5%(b)	0.4 x
		VIT	0.9	4%(b)	0.4 x
		82	0.9	1%(c)	1.0 x

Autoradiographs like those illustrated in Figs.1 and 2 were prepared using nucleoids from each cell-line, scanned using a microdensitometer, peak heights measured and the relative intensities of the bands determined by reference to similar bands obtained with varying weights of total DNA. (a), taken from Fig. 1; (b), taken from Fig 2; (c), the filter used for Fig. 2 of Cook et al., (12) was reprobed. pMA 51 contains the same insert as pMA 50 (which is described in ref. 9) subcloned in the vector pMA 300 (9).

with Eco Rl and then 5 µg of each ligated with 2 µg of the appropriate vector. Finally, the ligation mixture was screened for plasmids that had gained the capacity to replicate autonomously in yeast cells. [All experimental details are given in the accompanying paper (9)]. If human ARS's are associated with the cage throughout the cell cycle, we would expect the cage-associated fraction to be very much richer in ARS's than total DNA. However, only 2 ARS's were detected in the cage-fraction, compared to 4 in an identical weight of total DNA. Although these numbers do not permit any confident conclusions, the cage fraction is clearly not greatly enriched in ARS's.

4 plasmids containing human ARS's (two derived from total HeLa DNA, one from the cage-associated fraction and another from total human placental DNA) have been recovered from yeast cells and characterized by Monteil et al. (9). The source and size of the human inserts in these various plasmids are summarized in Table 1. We used 'nick-

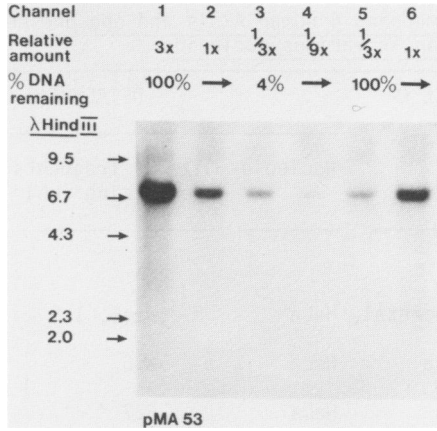

Fig 1. Detachment mapping the ARS contained in pMA53 in HeLa nucleoids. Samples of total DNA (100% remaining) and DNA that resisted detachment by Eco R1 (4% remaining; channels 3,4) were completely redigested with Eco RI and various amounts (3x, 1x, 1/3x, 1/9x) applied to a gel. After electrophoresis, blotting and hybridization with 'nick-translated' pMA53 DNA, an autoradiograph was prepared and photographed. The sizes of marker fragments of λ DNA cut with Hind III are given in kilobases.

translated' DNA from these plasmids as probes to map the positions of the corresponding human ARS's in the loops of nucleoid DNA using our established method (11, 12).

DNA is detached from nucleoid cages using Eco R1: then cages - and any associated DNA - are sedimented free of detached DNA. The cage-associated DNA is purified and completely redigested with Eco R1. Known weights of this DNA are resolved into discrete fragments by gel electrophoresis, 'blotted' on to a filter, hybridized with the 'nick-translated' ARS probe and the relative amount of hybridizing sequence determined by autoradiography. Following the first partial digestion, sequences lying close to the point of attachment tend to cosediment with cages and so be present in relatively greater abundance on the filter: therefore, they yield bands of greater intensity on autoradiography. In contrast, those lying out in the loops are readily detached and so are depleted in the pelleted fraction. The degree of enrichment or depletion is determined by reference to known amounts of total DNA run in adjacent channels in the gel.

Fig. 1 illustrates the mapping of the human ARS carried in pMA53 using HeLa nucleoids from unsynchronized HeLa cells. Control DNA,

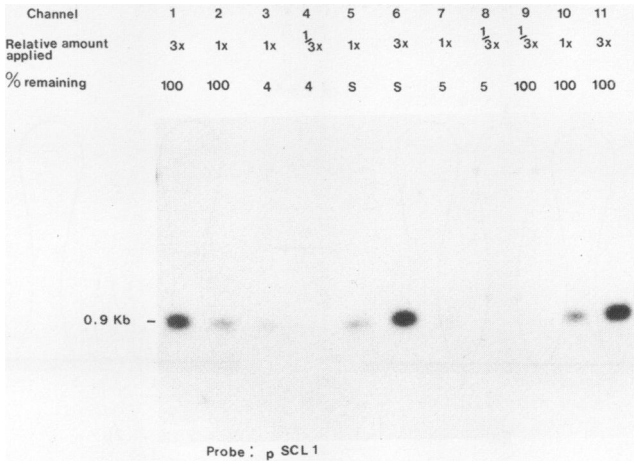

Fig. 2. Detachment mapping a candidate for a terminus of replication in VII nucleoids

Total DNA (100%; channels 1,2,9,10,11), DNA which was detached by Eco R1 (channels 5,6) or which resisted detachment (4% remaining, channels 3,4; 5% remaining, channels 7,8) were applied to the gel. The detached DNA used in channels 5 and 6 was purified from the supernatant (S) above the pellets used for channels 3 and 4 and redigested with Eco R1. Channels 7 and 8: nucleoids were pre-treated with ribonuclease to detach >96% of nascent RNA prior to the first digestion with Eco R1. [For further details see Fig. 4, Cook et al., (12), where an autoradiograph of this filter using an avian sarcoma virus probe is given: the ASV sequences were enriched >3x. Enrichment of ASV sequences and depletion of the 0.9 kb fragment on the same filter rules out the trivial objection that incorrect weights of DNA have been applied to the filter].

undigested during the first partial digestion, yields one 6.7 kb band when subsequently digested completely with Eco R1 and hybridized with 'nick-translated' pMA53 DNA (Fig. 1, channel 2: i.e. 100% remaining). When 3 times, or $^{1}/_{3}$ times, this amount of DNA is applied to the gel the bands are correspondingly stronger or weaker (Fig 1, channels 1 and 5). The duplicate channels (2 and 6) of 1 times the control amount of DNA - which are included in all experiments - illustrate the uniformity of blotting and hybridization. If these nucleoids are partially digested to detach all but 4% of their DNA and then this DNA is purified and completely fragmented with Eco R1, the band obtained subsequently is slightly less intense than that obtained with an equal weight of control DNA (Fig. 1, compare channels 3 and 5). As we know that some sequences can be enriched 8x in HeLa nucleoids and 18 x in rat nucleoids (11, 12) we conclude that the 6.7 kb sequence cannot lie as close to the

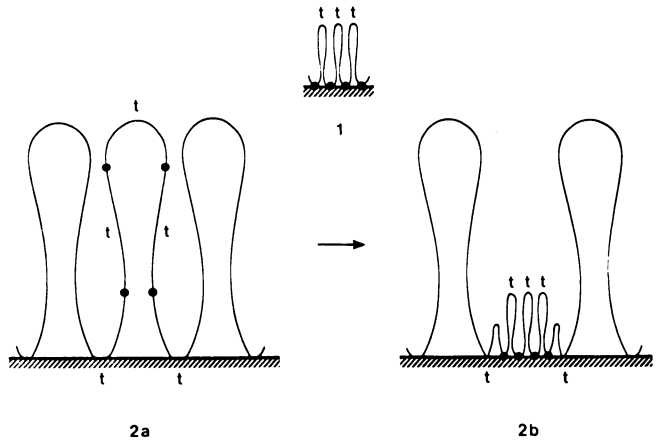

Fig. 3. 2 models for the organization during most of interphase of sequences involved in the initiation and termination of replication
(1) Three loops are attached by origins (●) to the nuclear matrix. (2a) Three structural loops of 220 kb and containing 4 replicons (~60 kb) are attached to the nuclear cage by specific sequences. 2 of the 5 termini (t) are attached but none of the origins. (2b) DNA synthesis begins by attachment of the 4 origins. 2a and 2b are consistent with the results given here and with previous data (5, 14). An alternative to 2 based on a loop equal in size to a replicon is that the initiating sequence is usually unattached but attaches during S phase. In this case, termini would usually be attached.

attachment point as these other sequences. The insert in pMA53 was originally derived from the cage-associated fraction so it must have associated non-specifically with a cage prior to cloning. None of the 3 other ARS's studied are enriched, so they too must lie out in the loops (Table 1).

Fig. 2 illustrates the mapping of a sequence that lies immediately adjacent to a candidate for a terminus of replication in the rat genome (10). The sequence, a 0.9 kb Eco R1 fragment, has been described in detail (13) and subcloned in plasmid pSCL1 (H. Manor, unpublished). 'Nick-translated' pSCL1 DNA was used with nucleoids from 2 different rat cell lines, VIT and 82 (12). Using VIT nucleoids, the 0.9 kb fragment is depleted in the pelleted fraction (Fig 2, channels 3,4; Table 1) and very slightly enriched in the detached supernatant fraction (Fig. 2, channels 5,6). Detaching nascent RNA has no effect on the sensitivity of the 0.9 kb fragment to subsequent detachment by Eco R1 (Fig. 2, channels 7,8). We conclude that this sequence usually lies remote from an attachment point.

DISCUSSION

Two simple models for the organization of sequences which initiate replication within loops of nuclear DNA have been suggested (Fig 3). In one, loops the size of replicons are attached by origins to the nuclear matrix during most of interphase (3, 4, 6). In the other, origins are usually remote from the sub-structure, attaching during S (5). Our reasons for preferring this model have been summarized elsewhere (5, 14). If we accept that these human ARS's are indeed sequences involved in initiating DNA synthesis, then both our findings are consistent with the latter model illustrated in Fig 3, 2a and b. First, the ARS concentration in the fraction resisting detachment is not greater than that in total DNA. Second, all 4 ARS's studied - and the potential termination sequence - map remote from attachment points. Perhaps transient attachment of these ARS's to the cage triggers DNA synthesis. We are currently investigating whether this is so, using synchronized cells.

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