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Replication origins are attached to the nuclear skeleton

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ABSTRACT

DNA fragments containing replication origins (oriDNA) were isolated from a chicken erythroblast cell line by a modified procedure of Zannis-Hadjopoulos et al. /1, 2/ and studied in the renaturation reaction driven by either total or nuclear matrix DNA (nmDNA) from the same cells or from mature erythrocytes. We found that the unique sequences of nmDNA from erythroblasts (5 kb long) represented a specific subset of sequences constituting about a quarter of total DNA unique sequences, while the erythrocyte nmDNA 5 kb fragments constitute only about one tenth of total unique DNA and all are recovered among erythroblast nmDNA. Virtually all oriDNA sequences are present in the fraction of erythrocyte nmDNA. Thereafter, the putative positions of replication origins within the α-globine gene domain have been mapped by hybridization experiments. They were found to coincide with the previously established positions of permanent sites of DNA attachment to the nuclear matrix.

INTRODUCTION

In the eucaryotic cell, DNA is organized into a series of loops attached to the nuclear matrix /4/. The latter is a proteinaceous structure that can be isolated from the nuclei after chromatin solubilization by nuclease treatment and high salt extraction /5, 6, 7/. The points of DNA attachment to the nuclear matrix are heterogeneous in nature. Different functions such as replication /7/, repair /8/ and transcription /9/ were shown to be realized at the nuclear matrix. The DNA attachments connected with these functions are commonly regarded as temporal or functional attachments. It has also been suggested that there are attachment points which do not depend on the nuclear activity /3, 10, 11/. An intriguing speculation is that these structural attachment points coincide with the

positions of replication origins. A model suggesting the association of replication origins with some intranuclear structures had been proposed by Dingman even before supercoiled loops were identified in eucaryotic DNA /12/. Several attempts have been made to test the model with conflicting results /13-16/.

Meanwhile, a method has been developed for the isolation of an eucaryotic DNA fraction enriched in replication origins (oriDNA) /1, 2/. In the present investigation, we compared the sequence specificity of this DNA fraction with that of nuclear matrix DNA (nmDNA) using two experimental approaches. First, we compared the renaturation kinetics of total DNA, oriDNA from the chicken erythroblast cell line and nmDNA from the same cells in reactions driven by total DNA, erythroblast cell line nmDNA and erythrocyte nmDNA. Second, the positions of replication origins within the domain of chicken α-globin genes were mapped with the aid of hybridization experiments and compared with the previously established positions of DNA attachments to the nuclear matrix /3/.

The results obtained in the both series of experiments agree with the idea that replication origins are associated with the nuclear skeleton and have the same positions as permanent attachment sites.

METHODS

Cell culture and labeling

A chicken erythroblast cell line was cultivated in the Dulbecco medium supplemented with 10% of fetal calf serum. For DNA labeling, [^{14}C]thymidine (0.2 μCi/ml) was added to the medium within 48 h. Pulse labeling was carried out for 5 min in a medium containing [^{3}H]deoxycytidine (15 μGi/ml) and bromodeoxyuridine (15 μg/ml).

Isolation of DNA enriched in replication origins (oriDNA)

A modification of the nascent DNA-strand extrusion method /1, 2/ was used. After pulse labeling, the cells were suspended in buffer containing 20 mM Tris-HCl, pH 7.5, 20 mM NaCl, 2.5 mM EDTA (TNE buffer). SDS was added to a final concentration of 0.5% and proteinase K up to 200 μg/ml. The mixture

was incubated for 6 h at 37°C. Then it was applied to a 5-20% sucrose gradient. Sucrose solutions were prepared in TNE buffer with a NaCl concentration increased to 500 mM. The gradients were spun in a Beckman SW-27 rotor for 16 h at 22,000 rpm and 10°C. The upper fractions (8-18S) enriched in pulse-labeled DNA were collected, the DNA was precipitated with ethanol and dissolved in a small volume of TNE buffer. The appropriate volumes of saturated CsCl, 2 M Tris-HCl, pH 7.5, 500 mM EDTA and 20% sarkosyl were added to obtain a CsCl solution ρ=1.74 g/cm^3 in TNE with 0.15% sarkosyl. Density centrifugation was performed in a Beckman Ti 50 rotor for 60 h at 40,000 rpm and 16°C. After the centrifugation, fractions containing extruded nascent DNA duplexes (ρ =1.82 g/cm^3) were combined and dialysed against TNE buffer. DNA was precipitated with ethanol, dissolved in TNE buffer and treated with RNase A (Worthington, 50 µg/ml) for 1 h at 25°C. After deproteinization and ethanol precipitation, the pellet was dissolved in an appropriate buffer and treated with nuclease S1 (PL; 5 U/ml) for 1 h at 37°C. Then density centrifugation in a CsCl solution ρ =1.74 g/cm^3 was repeated twice. Upon the last centrifugation, only a peak composed of extruded nascent duplexes could be detected in the gradient. The respective fractions were combined and dialysed against TNE buffer. DNA was precipitated with ethanol, dissolved in TNE buffer and additionally fractionated according to fragment sizes in a 5-20% sucrose gradient. The gradients were spun in a Beckman SW-60 Ti rotor for 3 h at 55,000 rpm and 16°C. Fractions containing DNA fragments of the appropriate sizes were collected, diluted with TNE to decrease the sucrose concentration, and DNA was precipitated with ethanol.

The 1-2 kb subfraction of oriDNA used in the hybridization experiments was additionally purified by preparative electrophoresis in 1% agarose gel.

Isolation of nmDNA

A method described in our previous paper /17/ was used to isolate nmDNA from a chicken erythroblast cell line and from mature erythrocytes. Briefly, the cells were lysed with NP-40 (0.5%) and Triton X-100 (0.1%) in TMG buffer (50 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 5% glycerol) containing also 1 mM $CuSO_4$.

The nuclei were washed three times with TMG buffer without $CuSO_4$ and suspended in TMG buffer (10^8 nuclei/ml). $CaCl_2$ (up to 2 mM), NaCl (up to 0.5 M) and a necessary amount of micrococcal nuclease were added. The suspension was incubated 5 min at 25°C. Then, after cooling, the fast sedimenting structures were pelleted and washed twice with TMG buffer containing 0.5 M NaCl and twice with TMG buffer containing 1 M NaCl. Finally, the preparation was suspended in TMG buffer containing 2 M NaCl and pelleted through a layer of 2 M NaCl-TMG buffer with an elevated glycerol concentration (10%). DNA was isolated from the nuclear matrices using proteinase-detergent extraction /18/.

Renaturation experiments

Renaturation experiments were carried out as described elsewhere /10/. The percentage of renatured DNA was determined with an aid of hydroxyapatite chromatography. Probes were labeled with [^{32}P]dCTP by nick translation. This procedure is known to yield some amount of artificial hairpin-like structures. The presence of these palindromes does not reflect the genuine properties of the investigated DNA. Therefore, the percentage of renatured DNA at $C_0t = 10^{-5}$ moles x l^{-1} x s (from 6 to 10% in all cases) was regarded as zero when renaturation curves were constructed. The actual amount of palindromes in the preparation taken for nick-translation was determined using self-renaturation of *in vivo* labeled DNA. It did not exceed 1.5% for each of the preparations. Before renaturation, all the DNA preparations were cleaved to ~200 nucleotide fragments by boiling in 0.3 M NaOH. The percentage of renaturation at $C_0t = 3 \times 10^4$ moles x l^{-1} x s in the reaction driven by total DNA was assumed to be 100% (actually, it varied from 85 to 90%). All the renaturation percentages at other C_0t values (in the reactions driven either by total DNA or by nmDNA) were normalized in accordance with the above assumption.

Hybridization experiments

Nick-translated DNA probes were hybridized to Southern filters as previously described /19/. Equal amounts (0.3 μg of DNA with a specific activity of 10^8 cpm/μg) of either total

DNA or oriDNA were hybridized to similar filters so that the intensity of hybridization signals could be compared.

Enzymatic reactions

Nick-translation, DNA end-labeling with polydeoxyribonucleotidyl transferase and digestion of DNA with restriction enzymes were carried out as was described /19/.

RESULTS

Isolation of the DNA fraction enriched in replication origins

OriDNA was isolated using a method based on the extrusion of nascent DNA strands from the replication loops /1, 2/. Cells were labeled for 48 h with [^{14}C]thymidine and then for

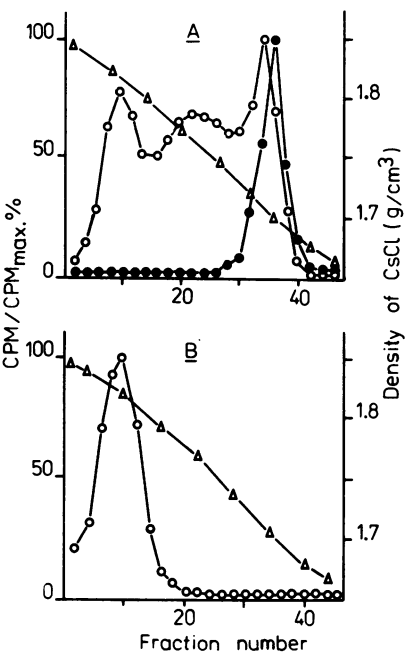

Fig.1. Distribution of long-term labeled DNA (closed circles) and pulse-labeled DNA (open circles) upon the first (A) and third (B) centrifugations of the preparation in a CsCl density gradient. The density curve is marked with triangles. After the third centrifugation (panel B) no long-term labeled DNA was detected in the gradient fractions. Thus, only the distribution of pulse-labeled DNA is presented.

5 min with [^{3}H]deoxycytidine and bromodeoxyuridine (BUdR). DNA was isolated, incubated under the conditions favourable for nascent chain extrusion (see Methods) and ultracentrifuged in a sucrose gradient. The slowly sedimenting fraction was recentrifuged in a CsCl density gradient (Fig. 1A). This step is crucial for oriDNA purification. First, the bulk of contaminating DNA fragments (practically all ^{14}C-labeled DNA) is removed, being banded at a density of 1.7 g/cm^3 as they do not contain BUdR. Second, only oriDNA is substituted with BUdR in the both strands according to the extrusion procedure /1, 2/ and therefore it is banded as a sharp peak with a density of 1.82 g/cm^3. Other replication intermediates and the products of the reparation reaction should contain BUdR in one strand only. They are recovered as a broad peak with an average density of about 1.76 g/cm^3 (Fig. 1A).

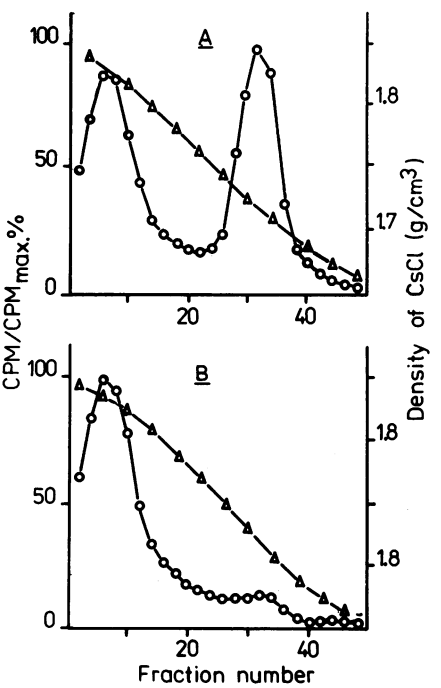

Fig. 2. Distribution of end-labeled DNA chains upon the centrifugation of DNA fragments with a buoyant density of 1.76 g/cm^3 (A) and 1.82 g/cm^3 (B) in an alkaline CsCl gradient. The density curve is marked with triangles.

This statement was confirmed in the following experiment. The DNA from the both above mentioned heavy peaks was collected and treated with S1 nuclease. Most of the pulse-labeled DNA was resistent to such a treatment in the both cases. Then the ends of the DNA chains were labeled with [α^{32}P] dideoxy ATP in the reaction catalyzed by terminal deoxyribonucleotidyl transferase and the distribution of the DNA chains in an alkaline CsCl density gradient was analyzed. About half of the end-labeled DNA from the 1.76 g/cm^3 peak was found at a density of 1.83 g/cm^3 while the other half banded at a density of ~1.72 g/cm^3 (Fig. 2A). Thus, the dsDNA fragments with a buoyant density of 1.76 g/cm^3 were composed of one parental and one nascent (containing BUdR) strand. In contrast, virtually all the end-labeled DNA from the 1.82 g/cm^3 peak was banded in an alkaline CsCl gradient at a density of 1.83 g/cm^3 as could be expected if this dsDNA was composed of two heavy nascent DNA chains (Fig. 2B).

In preparative experiments, the DNA with a buoyant density of 1.82 g/cm^3 was collected and treated with RNase A and with nuclease S1 to eliminate transcription intermediates and Okasaki fragments (single-stranded DNAs). Then the preparation was twice recentrifuged in a CsCl density gradient to eliminate traces of DNA substituted with BUdR in one strand (Fig. 1B).

Now, the preparation was once again fractionated by ultracentrifugation in a sucrose gradient and fragments of various sizes were collected. The 3-6 kb subfraction was used in renaturation experiments. The smaller fragments (1-2 kb) were taken for blot hybridization experiments. They were additionally purified by agarose gel electrophoresis to eliminate longer fragments.

Renaturation of total DNA, oriDNA and nmDNA in reactions driven by nmDNA and by total DNA

For renaturation experiments, nmDNA with an average size of ~ 5 kb was isolated from a chicken erythroblast cell line and also from mature erythrocytes. Both preparations of nmDNA as well as total chicken DNA were cleaved to ~ 200 nucleotide fragments and used to drive the renaturation of the following nick-translated DNA probes: (1) nmDNA from the erythroblast

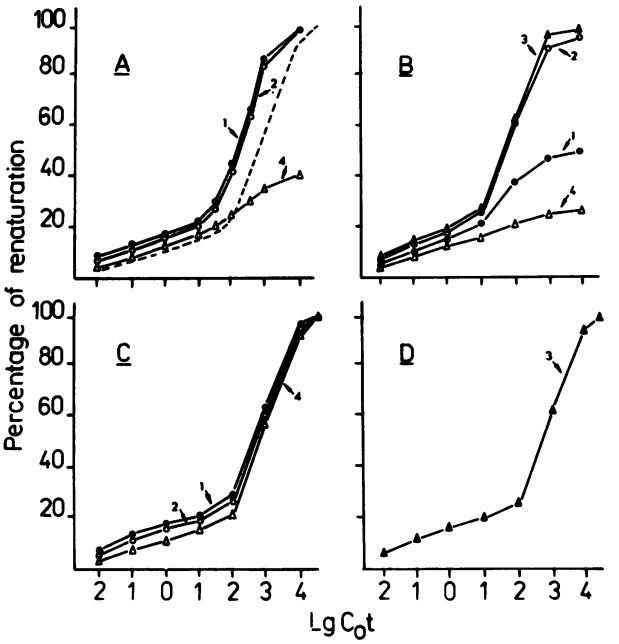

Fig. 3. Renaturation of different probes in the reactions driven by erythroblast cell line nmDNA (A), erythrocyte nmDNA (B) and total DNA (C, D).
The renaturation curves are marked with closed circles for the erythroblast cell line nmDNA probe (curves N1), with open circles for the oriDNA probe (curves N2), with closed triangles for the erythrocyte nmDNA probe (curves N3), and with open triangles for the total DNA probe (curves N4). The dash line in panel A is a renaturation curve for the total DNA probe in the reaction driven by total DNA. For details of C_0t curve construction, see Methods.

cell line, (2) oriDNA from the erythroblast cell line, (3) nmDNA from erythrocytes, and (4) total chicken DNA. The renaturation curves are shown in Fig. 3.

One can see that the preparations of nmDNA contain specific subsets of sequences present in total DNA.

First, only a fraction of total DNA is reannealed in the presence of a large excess of nmDNA at $C_0t = 10^4$. About 25% of total DNA unique sequences renatures in the reaction driven by nmDNA from erythroblast cells and about 10% in the reaction driven by nmDNA from erythrocytes (curve 4 in Figs. 3A and 3B, respectively). In contrast, 100% renaturation was obtained in

the reactions driven by all homologous DNAs (curve 1 in Fig.3A, curve 3 in Fig. 3B, and curve 4 in Fig. 3C) as well as in nmDNA annealing driven by total DNA (curves 1 and 3 in Figs. 3C, D) and in erythrocyte nmDNA annealing driven by erythroblast nmDNA (not shown). On the other hand, only 35% of erythroblast nmDNA unique sequences renatures in the presence of an erythrocyte nmDNA excess (curve 1 in Fig. 3B).

Thus, the unique sequences present in erythrocyte nmDNA with an average length of ~ 5 kb comprise about one third of erythroblast nmDNA unique sequences and one tenth of total unique DNA sequences.

Second, the half-renaturation C_0t values ($C_0t_{1/2}$) for unique DNA sequences in the reaction driven by nmDNAs are lower than in the case of total DNA ($\sim 10^3$) being equal to $\sim 10^2$ (erythrocyte nmDNA) and $\sim 2.5 \times 10^2$ (erythroblast nmDNA). Thus, the complexity of unique sequences in nmDNA is ca. ten and four times lower than that in total DNA.

Considering these results, we reannealed oriDNA in the presence of the same drivers. In all the three cases, oriDNA renaturation was virtually complete (curves 2 in Figs.3A,B,D).

Thus, all oriDNA is recovered among the sequences present in nmDNA either from erythroblast cells or from erythrocytes. In other words, even a small subset of total DNA ($\sim 10\%$) present in erythrocyte nmDNA includes all the replication origins isolated from erythroblast culture cells.

As was shown previously /3/, chicken erythroblast nmDNA contains both the sequences permanently attached to the nuclear skeleton and the transcribing DNA while erythrocyte nmDNA contains only the former sequences. Thus, replication origins belong to those sequences which are permanently attached to the nuclear skeleton and their attachment does not depend on the nuclear functional activity.

Mapping the positions of replication origins within the domain of chicken α-globin genes

We have recently mapped the preferential positions of DNA attachments to the nuclear skeleton within the domain of chicken α-globin genes /3/. The distribution of the attachment points was found to depend on the functional state

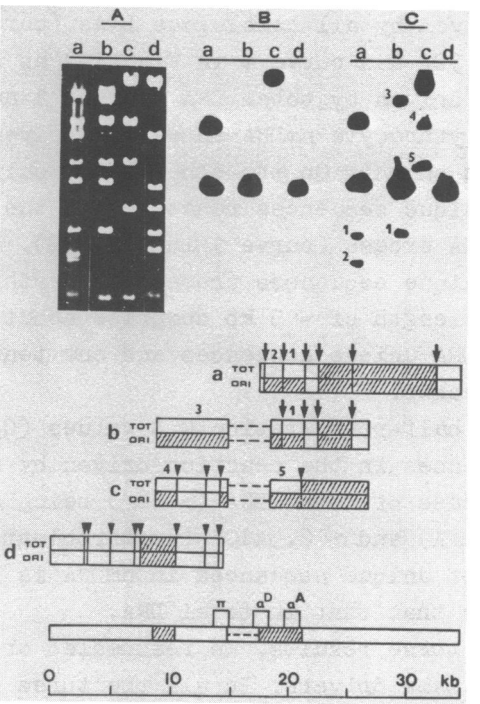

Fig. 4. Southern blot hybridization of oriDNA and total DNA with cloned fragments of the chicken α-globin gene domain.

Panel A. Electrophoretic distribution of restriction fragments of recombinant DNA used for preparation of Southern blots (ethidium bromide staining).

Panel B. Hybridization to nick-translated total DNA.

Panel C. Hybridization to nick-translated oriDNA.

The following DNA fragments were used to prepare Southern blots:

λCαG2 DNA - restricted with BamHI (slot a),
λCαG5 del. DNA - restricted with BamHI (slot b),
λCαG5 del. DNA - restricted with HindIII (slot c),
λCαG6 DNA - restricted with HindIII (slot d).

The maps for recombinant DNA insertions are given below the autoradiographs. The positions of BamHI and HindIII restriction sites are indicated with tailed and tailless arrows, respectively. The dash line in between the two parts of the λCαG5 del. map designates the position of a deletion. Fragments separated in the maps with a dash line are physically linked in the insertion.

In the upper part of each map, fragments detected upon hybridization to the total DNA probe are marked with sloping lines. In the lower part, fragments detected upon hybridization to the oriDNA probe are marked in the same way. Fragments which hybridized to oriDNA but not to total DNA are also indicated with numbers (1-5), both in the autoradiographs (panel C) and in the maps. Note that fragments 3 and 4 are composed of the insertion and the vector sequences.

The results of hybridization experiments are summarized in the map of the α-globin gene domain. Here, the sloping lines indicate the putative positions of replication origins (the regions highly enriched in oriDNA). A part of fragment N5 located to the left of the deleted region is not marked as being enriched in oriDNA since there is no evidence for independent hybridization of this area to the oriDNA probe.

of the globin genes. The entire transcriptionally active area was attached to the nuclear matrix in erythroblast nuclei. In fully repressed erythrocyte nuclei, in contrast, two distinct attachment points were observed. The major attachment point was located 3 kb upstream from the π gene and the minor one near the α^A globin gene.

To find the positions of replication origins, we hybridized nick-translated oriDNA to the restriction fragments of cloned α-globin gene DNA fractionated by electrophoresis and transferred onto nitrocellulose filters. In control experiments, nick-translated total DNA was hybridized to similar blots. $\lambda C\alpha G6$, $\lambda C\alpha G2$ of Engel and Dodson /20/ and $\lambda C\alpha G5$ del. were recombinant globin clones used in the hybridization experiments. $\lambda C\alpha G5$ del. was a spontaneous deletion of the original $\lambda C\alpha G5$ clone of Engel and Dodson. This deletion mutant was obtained and characterized by Scherrer and co-workers (unpublished).

The physical maps for insertions of the above clones and the results of the hybridization experiments are shown in Fig. 4. Both total and oriDNA probes gave strong hybridization signals with the restriction fragments containing repetitive sequences. The latter seemed to be equally represented in total DNA and in oriDNA, an observation which was verified in dot-blot hybridization experiments (not shown). The oriDNA probe also hybridized strongly to the restriction fragments containing unique sequences between 3 and 4.5 kb upstream from the π gene and near the α^A gene (in fact, in between the α^A and α^D genes, as both the 1.8 kb (N1) and 1.6 kb (N2) BamHI fragments of the $\lambda C\alpha G2$ clone gave signals of a comparable intensity upon hybridization with the 1-2 kb size fraction of oriDNA) (Fig. 4C, line a). Thus, exactly the same areas are enriched in oriDNA and nmDNA from erythrocytes. The only dif-

ference is in the relative intensity of hybridization. The both aforementioned regions seem to be equally represented in oriDNA (Fig. 4) while the sequences located upstream from the π gene are more abundant in erythrocyte nmDNA /3/.

Considering the results represented in Fig. 4, one should keep in mind that restriction fragments of the chicken α-globin gene domain showing the preferential hybridization with oriDNA (fragments N1 to N5 in Fig. 4) are composed of single-copy DNA sequences. Unique bands were observed upon hybridization of these fragments to total DNA digested by apropriate restriction enzymes /21/. It is also important that only some single-copy DNA fragments of the α-globin gene domain bind detectable amounts of the oriDNA probe. For example, no one of the single-copy DNA fragments generated upon digestion of $\lambda C\alpha G6$ insertion with HindIII was detected after hybridization of the corresponding Southern filter with oriDNA (Fig.4, lines d and scheme d). Similarly no hybridization of oriDNA with 1.0 kb BamHI fragment of $\lambda C\alpha G2$ insertion was observed while the neighbouring 1.8 kb BamHI fragment (N1) gave prominent hybridization signal.

If the signals of comparable intensity were observed upon hybridization of the oriDNA probe with all single-copy DNA fragments tested, one might explain the result by simple overexposition of the autoradiograph or by higher specific activity of the oriDNA probe as compare to the total DNA probe. However, the specific hybridization pattern observed in experiments represented in Fig. 4c (i.e. intensive hybridization of oriDNA with some single-copy DNA fragments and absence of hybridization with the others)is more likely to reflect the non-random representation of different regions of the α-globin gene domain in oriDNA.

Unfortunately, conclusions following from the analysis of autoradiograms represented in Fig. 4 are rather qualitative than quantitative. To estimate the level of enrichment of ori DNA with unique sequences located 3 kb upstream from π gene the other experiment was done. Now oriDNA (0.2 μg) and total DNA (different amounts from 0.2 μg to 2 μg) were immobilized on nylon filters and hybridized to nick-translated cloned DNA.

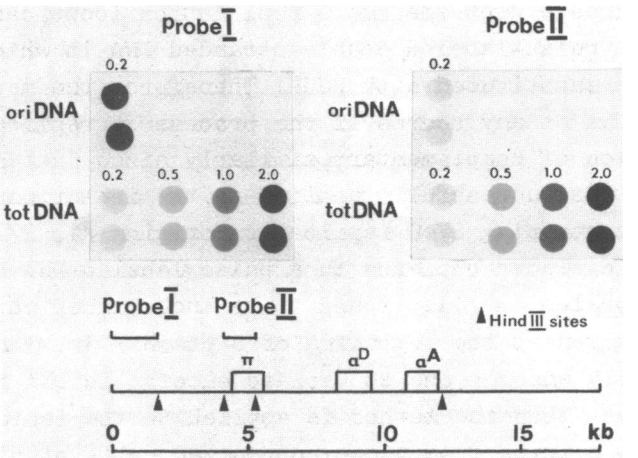

Fig. 5. Hybridization of two different fragments of λCαG5 insertion to oriDNA and total DNA immobilized on nylon filters.
The figures above the each pair of spots indicate the amount of DNA immobilized on corresponding filters. Below the autoradiogram, a scheme demonstrating the positions within the λCαG5 insertion of restriction fragments used as probes is presented.

Two hybridization probes were prepared. These are the 1.8 kb EcoRI-HindIII fragment represented the left end of λCαG5 insertion (probe I) and 1.1 kb HindIII fragment of λCαG5 DNA including π gene (probe II). The dot-blot hybridization experiment was done exactly as described /22/ except that Hybond filter (Amersham) was used instead of Gene Screen. This substitution was due to the higher capacity of Hybond filters as compare to Gene Screen. The results are shown in Fig. 5. One may see that the sequences of probe II are about equally represented on oriDNA and total DNA. However, the sequences of probe I are at least ten times more abundant in oriDNA as compare to total DNA. This confirms the conclusions of mapping experiments represented in Fig. 4.

DISCUSSION

The validity of the used method of oriDNA preparation

The method of nascent DNA-strand extrusion /1, 2/ used in our experiments is based on the expected physical properties of replication origins in isolated DNA. Neither of the known

DNA structures except the small replication loops can give rise to the pulse-labeled double-stranded DNA in which both strands are substituted with BUdR. Therefore, the method should work with DNA of any source if the process of replication leads to production of complementary, similarly sized nascent strands. The method was successfully applied to the isolation of DNA fragments containing SV40 replication origins /1, 2/. The proportion of extruded duplexes in a pulse labeled DNA of Physarum polycephalum was also found to be much higher when the pulse was given at the beginning of S-phase /23/. Thus, the method really enables one to isolate eucaryotic DNA replication origins. When the method is applied to the isolation of replication origins from non-synchronized cells of higher eucaryotes, the main technical problem is to separate extruded duplexes from contaminating fragments of pulse-labeled DNA. The proportion of extruded duplexes in pulse-labeled DNA should be extremely low due to the large size of replicons. However, several cycles of density gradient centrifugation makes it possible to purify extruded DNA duplexes. Obviously, replication forks and DNA repair intermediates should be distributed more or less randomly throughout DNA. Indeed, hybridization experiments with the 1.76 g/cm^3 peak taken as a probe showed that the distribution was quite random (data not shown).

In contrast, the ca. 5 kb DNA fragments containing putative replication origins isolated in our work are not randomly distributed but represent a specific subset of sequences comprising not more than 10% of the total unique DNA.

Replication origins are located at or closely to the nuclear skeleton

Our results are clearly indicative of a close association between replication origins and the nuclear skeleton. Unique nmDNA sequences represent only a fraction of total unique DNA (from 1/4 to 1/10 depending on cells used) and they include all unique sequences present in oriDNA. As ca. 5 kb DNA fragments were used, the distance between the replication origin and the site of DNA attachment to the nuclear skeleton cannot exceed a few kb. However, the 100% renaturation of oriDNA driven by nmDNA suggests (though does not prove) the coincidence of

these two types of sequences.

In this respect, it should be pointed out that the same results have been obtained in experiments with the α-globin gene domain where the size of oriDNA and nmDNA was lower.

The use of rather long DNA fragments did not allow us to analyse the nature of replication origins or attachment sites themselves. Really, the renaturation curves for all the four probes (total DNA, oriDNA, nmDNA from two sources) in reactions driven by total DNA were rather similar although nmDNA and oriDNA were slightly enriched in repetitive sequences. However, the obvious advantages of our approach were that (i) the comparison of unique sequences made it possible to arrive at unequivocal conclusions about the sequence coincidence, and (ii) really active and potential replication origins could easily be discriminated even if both were represented by the same repetitive sequences. Indeed, in this case, the unique sequences surrounding active and potential replication origins should still be different.

Our results agree with the previous observations of Wanka and collaborators /13, 14/ who used a quite different approach to study the relationship between the distribution of replication origins and the points of DNA attachment to the nuclear skeleton. However, due to the technical limitations in their approach, the above authors were able to study only a subset of replication origins initiating at the beginning of S-phase. In contrast, we investigated the whole population of replication origins.

Todorova and Russev /15/ have recently published their results suggesting a random distribution of replication origins versus DNA attachments to the nuclear matrix. The strategy of their experiments was similar to that described here. However, they used another procedure /24/ to isolate a DNA fraction which is enriched in replication origins. DNA strands were cross-linked with trioxalen in the living cell and then short nascent DNA fragments located in between the cross-links were collected. Two possible factors may account for the fact that our results are inconsistent with those of Todorova and Russev /15/. First, these authors did not use density gradient centri-

fugation to purify oriDNA. Consequently, their preparations could be contaminated with fragments of total DNA. Second, in general, the efficiency of the isolation procedure used by Todorova and Russev remains undocumented. It is not obvious that the nascent DNA fragments found in between crosslinks are indeed initiated in vivo from the appropriate origins. Movements of the replication forks may be arrested by crosslinks but an abnormal initiation may occur immediately after the crosslinks. White and Dixon /25/ have observed precisely this situation when they studied the replication of SV40 DNA containing thymidine dimers. It has also been found that DNA crosslinking by psoralen decreased the replicon size in Pisum sativum cells probably due to the activation of abnormal initiation sites /26/. We have recently investigated the sequence specificity of a DNA fraction obtained from cells lytically infected with SV40 and subjected to the trioxalen crosslinking procedure. No preferential hybridization of this DNA fraction with restriction fragments of SV40 DNA containing the replication origin was observed /27/.

Cook and Lang /16/ examined the cellular distribution of specific mammalian DNA sequences that could function as ARS in yeasts. These sequences were not rich in the matrix DNA fraction. However, there is no evidence that yeast ARS sequences can function as proper replication origins in higher eucaryotes. It appears that experiments with cloned DNA sequences, which can autonomously replicate in mammalian cells, are necessary to analyse properly the distribution of replication origins using the approach of Cook and Lang.

Heterogeneity of DNA interactions with the nuclear skeleton and association of replication origins with permanent attachment sites

It becomes evident that DNA interactions with the nuclear skeleton are heterogeneous in nature. In particular, permanent attachment sites and associations originating in connection with the transcription have been identified in the chicken α-globin gene domain /3/. They were revealed when the sequence specificity of erythroblast nmDNA was compared with that of nmDNA from functionally inactive erythrocyte nuclei.

Here, we found that the number of DNA attachments to the nuclear skeleton in erythrocytes is about three times as low as in erythroblasts, the difference probably being due to the absence of transcription-dependent associations from erythrocytes. On the other hand, all attachments present in erythrocytes can also be detected in erythroblasts, i.e. they represent permanent DNA interactions with the nuclear skeleton.

This system was used to find out what kind of attachment sites coincides with replication origins. We found that these are permanent attachments present in erythrocytes inactive both in transcription and replication. The same conclusion was drawn from experiments with total unique sequences and with unique sequences of the α-globin gene domain.

The fact that replication origins are stably associated with the nuclear skeleton even in the absence of replication suggests that such an association cannot be attributed to aggregation of the replicative machinery in a high-salt solution.

The high-salt extraction method for nuclear matrix isolation was criticized recently since it induces aggregation and rearrangement of attachment sequences, and a low-salt detergent technique was proposed instead /28/. However, the both procedures enable identification of the same area upstream from the 5'-end of D. melanogaster HSP70 gene as being attached to the nuclear skeleton /28, 29/. The sequence specificity of nmDNA observed in our renaturation experiments (see Fig. 3) also demonstrates the validity of the high-salt isolation method used in the present investigation. Probably, the difference between the detergent and high-salt methods is that the latter leads to association of the whole transcribing DNA area including RNA polymerase - DNA complexes with the nuclear skeleton while the former method caused binding only of ends of active genes, possibly through topoisomerase II /30/.

Summarizing one may conclude that all oriDNA sequences are included in the fraction of most stable nmDNA recovered in inactive erythrocyte cells. These sequences may constitute a significant part of a permanent nmDNA although it is possible that nmDNA contains some other sequences even in erythrocyte cells. The question is under investigation now.

Abbreviations: nmDNA, nuclear matrix DNA; oriDNA, DNA fraction enriched in replication origins.

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