LATE REPLICATION OF THE DNA ASSOCIATED WITH THE NUCLEAR MEMBRANE

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1. Introduction

There is considerable light and electron microscopic evidence for the attachment of chromosomal DNA to the nuclear membrane [1, 2]. Furthermore, several of the reported nuclear membrane prepararations contain DNA [3–6]. It is possible, therefore, that a DNA replicating complex, involving the nuclear membrane, may exist in eukaryotes, analogous to the involvement of the cell membrane in bacterial DNA synthesis. Experiments on nuclei from regenerating rat liver, claiming to support this idea, have been published recently [6]. Similar suggestions have also been made from labelling studies on putative DNA-membrane complexes [7, 8].

In the work described here, we have isolated nuclei and nuclear membranes, following pulse labelling with ^{3}H-thymidine, from regenerating rat liver at various times during the first (synchronous) S phase after partial hepatectomy. The results show the nuclear membrane DNA to be predominantly replicated in the latter half of S phase. The evidence argues against the nuclear membrane being the site of all nuclear DNA synthesis.

2. Materials and methods

Wistar rats of body weight 150–200 g were used throughout. In individual experiments, they were of the same weight to within ± 5 g. They were starved for 12 hr before operation.

^{3}H-thymidine-6-T (26 Ci/mmole) was obtained from the Radiochemical Centre, Amersham and electrophoretically pure DNase from Sigma.

2.1. *Surgical procedure and injections*

Partial hepatectomies (approximately 60%) were carried out as previously [9] at different times before killing. After the operation the animals were returned to a controlled environment, with a 12 hr dark period from 7 pm–7 am. At approximately 9.15 am all the animals were injected intraperitoneally with 20 μCi per animal of ^{3}H-thymidine and killed exactly 15 min later.

2.2. *Preparation of nuclei and nuclear membranes*

Nuclei were prepared from the liver remnants as described [9] and finally washed with 0.25 M sucrose, 1 mM $MgCl_2$ pH 7.4. The resulting nuclei were free of other particles when examined by phase contrast and electron microscopy.

Nuclear membranes were prepared by a method which involves the release and solubilisation of the nucleoli and chromatin [10]. This entailed digestion of the nuclei with a low level of DNAse (1 μg DNAse per 2 mg nuclear protein per ml) in 0.01 M tris HCl, 10^{-4} M $MgCl_2$, pH 8.5. Membranes were pelleted, after one wash, at 38,000 *g* for 15 min. The method for isolating nuclear membranes was designed to give a preparation in which a small amount of DNA remained associated with the nuclear membrane. However, more extensive digestion with DNAse did remove virtually all of the DNA from the preparation.

The final membrane pellet used in these studies contained 50–60% of the nuclear phospholipid phosphorus (16 ± 3 μg per mg protein, cf. [11]) and 1–2% of the nuclear DNA. Examination of the preparation by phase contrast microscopy showed it to consist of nuclear ghosts and large fragments of these, and to be

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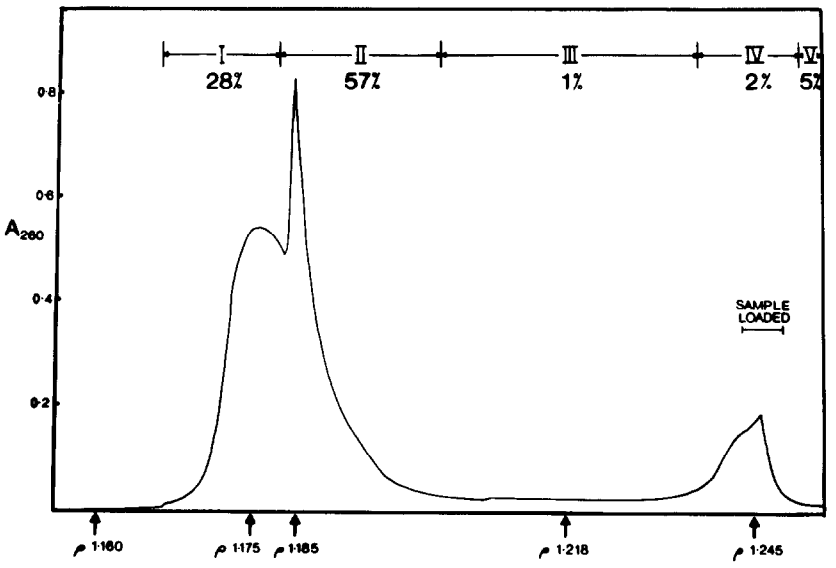

Fig. 1. The recovery and specific activity of membrane associated DNA from a continuous sucrose density gradient. Nuclei and nuclear membranes were prepared from rat livers 24 hr after 67% partial hepatectomy. Nuclear membranes were loaded as indicated (zone IV) and centrifuged as described in the Methods section. Specific activities (^{3}H cpm/mg DNA) were as follows: nuclei 84,020; nuclear membranes, as loaded 48,660, zone I 47,600, zone II 44,410. The figures represent the recovery of DNA in the various zones, as a percent of that loaded. Densities were determined at room temperature using an Abbé refractometer.

virtually free of intact nuclei. Electron microscopy (both negative staining and stained ultrathin sections) showed the presence of the typical double-layered membrane of the nucleus with ribosomes attached to one layer, nuclear pores and negligible amounts of adhering material.

2.3. *Density gradient centrifugation*

Continuous and discontinuous sucrose gradients, the latter in steps of 0.02 g/cc, were made over the density range 1.16–1.24 g/cc in a buffer containing 10 mM tris-HCl, 10 mM NaCl, 1 mM EDTA and 5 mM 2-mercaptoethanol pH 7.4. Nuclear membrane pellets, prepared as described were either resuspended in the above buffer and centrifuged into the gradient, or sucrose added to a density of 1.26 g/cc and loaded under the gradient. Gradients were centrifuged in the Spinco SW 25.1 rotor at 50,000 *g*, usually for 14 hr at 4°.

2.4. *Radioactive counting*

Samples for radioactive counting were precipitated with ice-cold 10% trichloroacetic acid, centrifuged, and the pellet either dissolved in 98% formic acid or extracted twice with 5% trichloroacetic acid (90°, 10 min) and aliquots of the solution counted in a liquid scintillation mixture at an efficiency of 20% (15 g BBOT, 200 g naphthalene, 1 l methylcellosolve, 1.5 l toluene).

2.5. *General analyses*

DNA was estimated by the diphenylamine reaction [12], protein by the Lowry procedure [13] and phospholipid phosphorus, after extraction of the phospholipid [14] and digestion with 60% w/w $HClO_4$, by the method of Chen et al. [15].

3. Results

We have attempted to show that the DNA in our preparations is, in fact, a closely associated component of the membrane. On both continuous and discontinuous density gradients, membranes from normal and regenerating liver nuclei were found to band in sucrose of density 1.17–1.19 g/cc. The bulk of the

DNA loaded was found to be associated with these membranes irrespective of loading at the top or bottom of the gradient. An experiment demonstrating this association is shown in fig. 1. The membranes are seen to band in two zones (I and II) although in some experiments only a single zone is observed. A very small pellet (zone V) observed in this experiment is usually absent; zone IV, at the position of loading, consists mainly of protein and is absent when membranes are layered over the gradient. The recovery of DNA in zones I–V was 93% of that loaded, of which 92.3% is in the nuclear membranes (zones I and II). The specific activity of the DNA in the recovered membranes was, for zone I, 98%, and for zone II, 91%, of that in the membranes loaded. In four other experiments using discontinuous gradients, the specific activity of the recovered membranes averaged 91% of that loaded (range, 85–97%; specific activities of loaded samples were 3,500, 23,270, 24,450, and 31,000 cpm/mg DNA respectively). Since the specific activity of the nuclear membranes in all five experiments was approximately half that of the bulk nuclear DNA (24 hr after partial hepatectomy; cf. fig. 2) the original membrane pellet loaded on the gradient cannot have been seriously contaminated with whole nuclei or nucleoplasmic DNA.

We have added partially DNAse digested radioactive chromatin from regenerating rat livers, 24 and 30 hr after operation, to unlabelled nuclei, and then prepared nuclear membranes from these. The results show that contamination of the nuclear membrane preparation by reassociation of released DNA accounts for only 5–7% of the recovered membrane DNA.

On the basis of the above observations we have determined the specific activities of the total nuclear DNA and of the nuclear membrane DNA at various times after partial hepatectomy. The results in individual experiments were normalised to the peak specific activity for the total nuclear DNA; they are shown in fig. 2. It can be seen that early in S phase, the specific activity of the nuclear membrane DNA is much lower than that of the total nuclear DNA, but towards the end of S phase the ratio of one to the other had increased to unity. In one experiment, by 30 hr, the nuclear membrane specific activity actually rose to 1.46 times that of the total nuclear DNA. Four separate experiments gave qualitatively similar results.

Our nuclear membrane preparations, therefore, appear to contain late replicating DNA. One further point concerning these results involves the possibility that the newly synthesised DNA of a membrane-bound replicating complex might be preferentially destroyed during the isolation process. However, the fact that the specific activities of the nuclear membrane DNA and total nuclear DNA, become identical later in S phase argues against this objection.

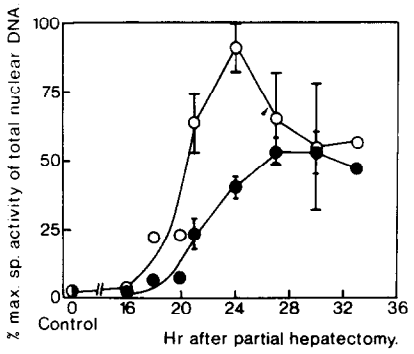

Fig. 2. Specific activities of the total nuclear (○) and nuclear membrane (•) DNA at various times after partial hepatectomy. The results from three experiments, covering the period from 16 to 33 hr after operation, are combined. In any single experiment, 5–6 groups of animals were used, one being a control group which was sham-operated. Animals were injected, 15 min before killing, with 20 μCi ^{3}H-thymidine. In each experiment values were normalised to the peak specific activity of the total nuclear DNA (18,200, 31,000 and 48,000 cpm/mg DNA for the three experiments). Each time point in each experiment was derived from the pooled livers of 4–6 animals. Points given as the means of 3 experiments are shown with standard errors; other values are single experimental points. In a fourth experiment (not shown) the peak of DNA synthesis has passed by 21 hr but the results were essentially similar.

After a 30′ pulse the specific activities of total nuclear and nuclear membrane DNA were approximately double those at 15′.

4. Discussion

These results, suggesting that the bulk of the DNA is not synthesised at the nuclear membrane are in agreement with recent autoradiographic studies. Working with synchronised cultures it was found, after short pulses with ^{3}H-thymidine, for both HELA cells [16] and Chinese hamster fibroblasts [17], that for most of S phase incorporation occurred throughout

the nucleus, but later in S phase the label at the nuclear membrane increased markedly.

Other studies have shown that heterochromatic DNA is replicated late in S phase [18]. Many of our own electron micrographs as well as those of others [17] show a preponderance of heterochromatin at the nuclear periphery. Taken together these observations suggest that we are detecting the replication of heterochromatic DNA in our nuclear membrane preparations. It is of interest to note that heterochromatin from mouse liver nuclei, for example, has been shown to contain substantial amounts of satellite DNA [19] while Tobia, Schildkraut and Maio [20] have reported this satellite to be a late replicating species in L cells.

We conclude that the bulk of nuclear DNA is probably not synthesised at the nuclear membrane.

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References

[1] D.E.Comings, Amer. J. Human Genet. 20 (1968) 440.
[2] D.E.Comings and T.A. Okada, Exptl. Cell Res. 63 (1970) 471.
[3] W.W.Franke, B.Deumling, B.Ermen, E.Jarasch and H. Kleinig, J. Cell Biol. 46 (1970) 379.
[4] I.B.Zbarsky, K.A.Perevoshchikova, L.N.Delektorskaya and V.V.Delektorsky, Nature 221 (1969) 257.
[5] R.Berezney, L.K.Funk and F.L.Crane, Biochim. Biophys. Acta 203 (1970) 531.
[6] N.S.Mizuno, C.E.Stoops and A.A.Sinha, Nature New Biol. 229 (1971) 22.
[7] F.Hanaoka and M.Yamada, Biochem. Biophys. Res. Commun. 42 (1971) 647.
[8] M.G.Ormerod and A.R.Lehman, Biochim. Biophys. Acta 228 (1971) 331.
[9] I.R.Johnston, A.P.Mathias, F.A.Pennington and D.Ridge, Biochem. J. 109 (1968) 127.
[10] R.R.Kay, D.Fraser and I.R.Johnston, in preparation.
[11] D.M.Kashnig and C.B.Kasper, J. Biol. Chem. 244 (1969) 3786.
[12] K.Burton, Biochem. J. 62 (1956) 315.
[13] O.H.Lowry, N.J.Rosebrough, A.L.Farr and R.J.Randall, J. Biol. Chem. 193 (1951) 265.
[14] E.G.Bligh and W.J.Dyer, Can. J. Biochem. Physiol. 37 (1959) 911.
[15] P.S.Chen, T.Y.Toribara and H.Warner, Anal. Chem. 28 (1956) 1756.
[16] R.A.Erlandson and E.De Harven, J. Cell Sci. 8 (1971) 353.
[17] C.A.Williams and C.H.Ockey, Exptl. Cell Res. 63 (1970) 365.
[18] A.Lima-de-Faria and H.Jaworska, Nature 217 (1968) 138.
[19] W.G.Yasmineh and J.J.Yunis, Exptl. Cell Res. 59 (1970) 69.
[20] A.M.Tobia, C.L.Schildkraut and J.J.Maio, J. Mol. Biol. 54 (1970) 499.