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TWO DISCRETE DNA REPLICATION INTERMEDIATES ARE FORMED IN MELPHALAN-TREATED CELLS

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Summary. In undisturbed cells one can detect a discrete population of 10 kb DNA replication intermediates, which indicates the existence of gaps spaced about 10 kb away from each other in the newly synthesized DNA. In melphalan-treated cells one can detect both 10kb and 20 kb DNA intermediates, indicating that in such cells the gaps present in a replicon are not filled at the same time which allows the detection of a molecule which is formed by the joining of two 10 kb DNA intermediates. In contrast in undisturbed cells it is likely that the filling of the gaps present in a replicon occurs at the same time which prevents the detection of a 20 kb DNA intermediate.

DNA replication in eukaryotic cells is a complex and poorly understood process. This is mainly due to the large size of the genome which makes it difficult to distinguish between different discrete DNA replication intermediates. Using a method of cell lysis in dilute alkali we have earlier shown that there exists a discrete 10 kb DNA replication intermediate in undisturbed cells (1). It is not possible to detect intermediates ranging in size from 10 kb up to 25 kb DNA. However in cells treated with the drug novobiocin it is possible to detect also a 20 kb DNA replication intermediate (2).

To obtain further evidence for a 20 kb DNA replication intermediate we have now analyzed cells where the replication is partially reduced by another drug, melphalan (l-phenylalanine), which is a bifunctional alkylator (3). In this paper we will show that when the DNA synthesis is slowed during a short time-period one can detect both 20 kb and 10 kb DNA populations. The results correlate well with a model for DNA replication in mammalian cells recently proposed by Taylor (4).

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Moreover the results are discussed in connection with the existence of gaps in the newly synthesized DNA, which is a prerequisite for the release of discrete 10 kb and 20 kb DNA populations.

MATERIALS AND METHODS

Cells, culture methods and labelling with ^{3}H-thymidine. A human melanoma cell line (CRL 1424), obtained from Flow Laboratories, U.K., were grown as monolayers at 37^{o}C in 5% CO_2 in air. The culture medium was Eagle's MEM with Earl's salts, containing 2mM l-glutamine, 10% fetal calf -serum and antibiotics. Routinely the culture medium was changed twice weekly and the cells passaged every 4 to 6 days (1).

For experiments the cells were seeded in small culture dishes (35x10mm) containing 3ml medium 24h before addition of 100 uCi ^{3}H-thymidine (22 Ci/mmol,Amersham Center, U.K.) and the incubation performed for the desired length of time.

Melphalan was dissolved in 15M ethanol containing 1.55M HCl (1ug melphalan/10ul) prior to the addition to the cell cultures. The acid ethanol-solution used to dissolve the melphalan was always added to the control cultures incubated in parallell with drug-treated cell cultures. The melphalan was a gift from Burroughs-Wellcome.

Cell lysis. The incubation medium was sucked off from the tissue culture dish and the cells rinsed twice in cold phosphate-buffered saline. Cell lysis was performed in the dark at 0^{o}C by the addition of 2.25ml of 0.03M NaOH. After 30 min the solution was neutralized by the addition of 0.9 ml 0.067 M HCl, 0.02M NaH_2PO_4(1). Finally the solution was made 0.5% with regard to SDS.

Gel electrophoresis. 0.75% agarose flat bed gels were made as described earlier (5). The labelled DNA was separated in the agarose gels using a LKB Multiphor electrophoretic system. DNA of known molecular weight, used as markers, were obtained from NEN. The gels were sliced in 1mm thick slices and the radioactivity was measured in a toluene-based scintillation fluid containing 3% Soluene 100, using a Packard scintillation counter.

DNA interstrand cross-linking. The experimental protocoll was adopted from (6). Cultures of 10^6 cells were labelled overnight with ^{3}H-thymidine. Control cells and melphalan-treated cells were lysed in 1 ml 6.8 M sodium perchlorate (pH 7) containing 1mM EDTA and 0.2% Sarkosyl. The mixture was heated at 75^{o}C for 5 min to denature the DNA and was then cooled in ice-water. To determine the extent of renaturation duplicate aliqoutes of each sample were treated with the single-strand specific nuclease S_1 (200 units/ml acetate buffer (pH 4.5) containing $ZnSO_4$) for 30 min at 37^{o}C after which cold TCA-precipitable activity was determined in a liquid scintillation counter.

RESULTS AND DISCUSSION

Due to the large size of the mammalian genome it has been difficult to distinguish between different discrete DNA replication intermediates. An approach that partially overcomes these problems is the so called unwinding technique (1). In this method the cells are lysed in dilute alkali which disrups the base pair structure of the DNA. However, the DNA strands cannot separate before enough time has elasped to allow unwinding of the DNA. When the solution is neutralized the high molecular weight DNA renatures and form double-stranded DNA whereas the smaller single-stranded DNA replication intermediates remain free in the solution. The replication intermediates can then be separated from the high molecular weight DNA by agarose gel electrophoresis.

Using this approach we have been able to show that one can release DNA replication intermediates from the parental DNA ranging in size from Okazaki-fragments up to 10 kb (1). The 10 kb DNA, which shows a discrete appearance in the gel electrophoresis, is formed by joining of Okazaki-fragments and is after a time-lag incorporated into high molecular weight DNA. Moreover in novobiocin-treated cells we have been able to detect both a 10 kb and 20 kb DNA intermediates in contrast to undisturbed cells where we have not been able to detect intermediates larger than 10 kb (2).

A prerequisite for the detection of the 10 kb DNA as a discrete population is that there exist gaps in the newly synthesiezed DNA spaced about every 10 kb (see Discussion in (1)). Since in undisturbed cells we did not detect intermediates which are larger than 10 kb, it seems likely that the filling of the gaps present in a replicon occurs approximately synchronously. A possible explanation for the presence of the 20 kb DNA intermediate in novobiocin-treated cells is, that the filling of the gaps present in a replicon does not occur at the same time, a situation in which it is possible to detect DNA fragments consisting of two joined 10 kb DNA intermediates.

If this interpretation is correct many agents that slow down DNA replication should be able to induce a change in the replication pattern which results in a disturbed synchrony of gap filling allowing the detection of both 20 kb and 10 kb DNA replication intermediates. To test this we have used a drug (melphalan) which has a completely different mode of affecting DNA replication than novobiocin.

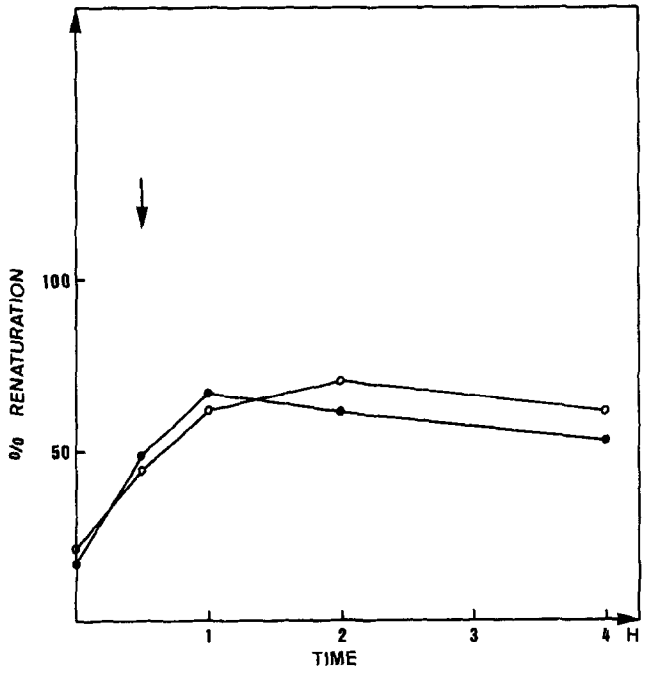
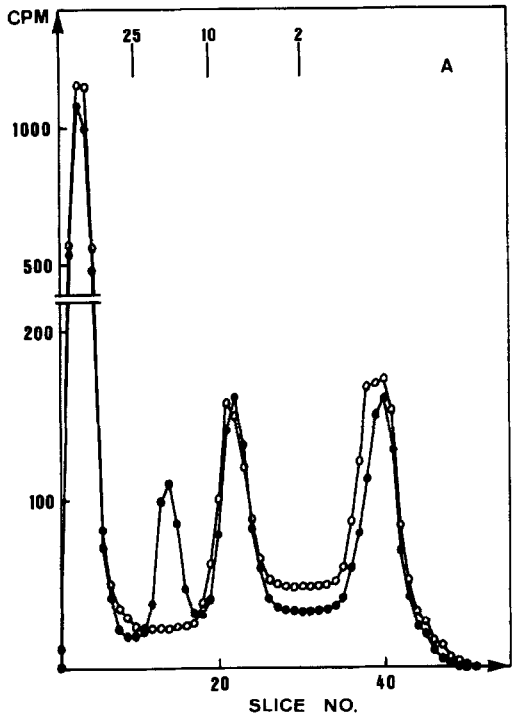


Fig. 1

DNA inter-strand cross-linking induced in melanoma cells by treatment with melphalan. Arrow indicates the time-point when melphalan (1ug/ml) was removed and the cells cultivated further in fresh medium. The background in untreated cells gives a value of about 20% renatured DNA. Two separate experiments are shown (-o-, -●-).

Melphalan is a bifunctional alkylator which gives rise to cross-links between the two DNA strands, within one strand and between DNA and protein (3). In human melanoma cells after a treatment with melphalan at a concentration of 1 ug/ml one can detect considerable cross-linking between the two DNA strands. The amount of the cross-linking continues to increase after the removal of the drug from the medium and reaches a maximum after 2 h (fig. 1). There is a reduction of total incorporation of tritiated thymidine into the DNA although the incorporation, 2 h after the treatment, still is more than 50% of the control value. The results are in agreement with earlier data showing that DNA replication in melanoma cells is rather insensitive to the existence of cross-linked DNA (6).

The melanoma cells were pulsed with tritiated thymidine for 5 min either immediately after the treatment with melphalan (fig. 2a) or after further cultivation in fresh medium for either 30min or 90 min (fig. 2b), 4h or 24h (fig. 2c). The cells were lysed in dilute alkali and the labelled DNA was analyzed by agarose gel electro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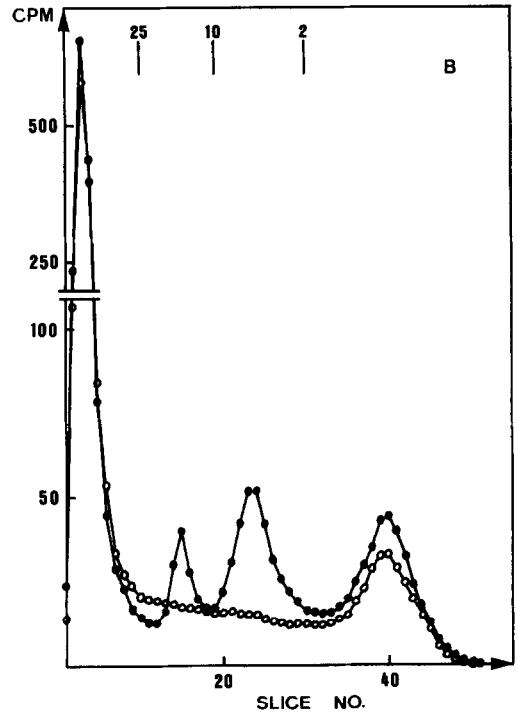
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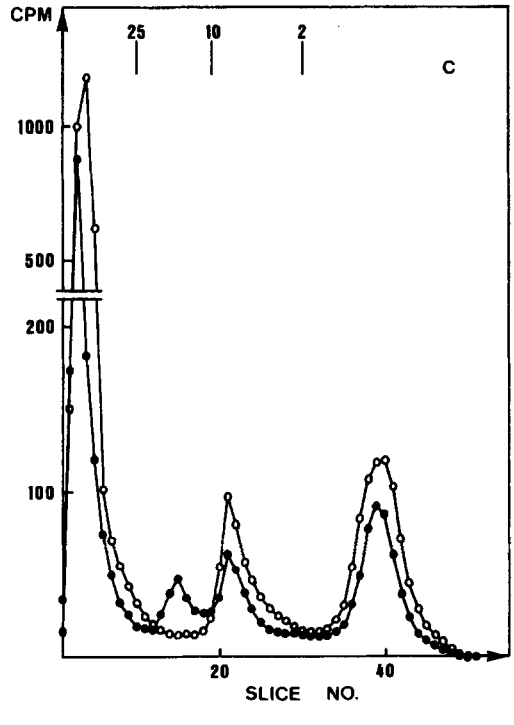
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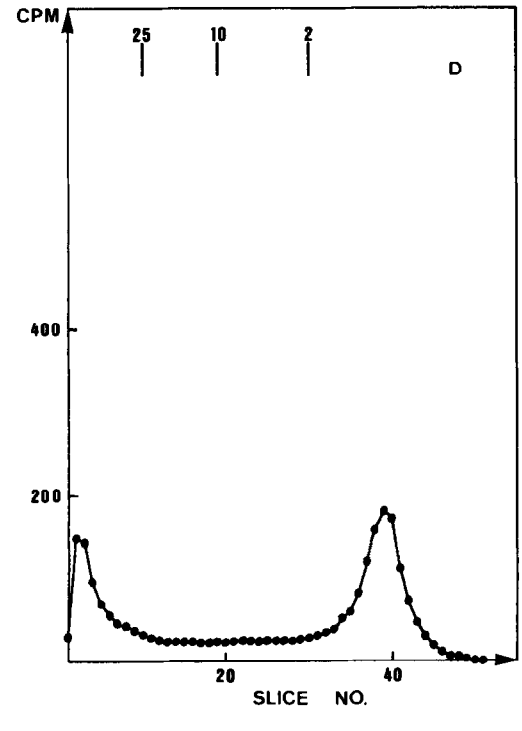
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phoresis. The results showed that immediately after the drug-treatment one can detect 20 kb DNA (slices 12-15), 10 kb DNA (slices 19-25) and Okazaki-fragments. Similar results were obtained 30 min after the drug-treatment. However, after 90 min one can detect only Okazaki-fragments, whereas after 4h it is possible once more to detect 10 kb DNA and a small amount of 20 kb DNA. 24h after drug-treatment the electrophoretic profile was very similar to the profile of DNA from undisturbed cells.

Experiments were also performed with the drug aphidicolin in order to show that both the 10 kb and 20 kb DNA populations are replicative intermediates and consequently depend on the function of DNA polymerase α. Aphidicolin is an inhibitor of DNA polymerase α, with almost no effect on polymerases β and γ (7). Melanoma cells were pretreated with aphidicolin for 60 min, treated with melphalan for 30 min in the presence of aphidicolin and then pulsed with tritiated thymidine for 5 min in the presence of aphidicolin but the absence of melphalan. The electrophoretic analysis now showed the absence of both 20 kb and 10 kb DNA (fig. 2d, cf fig. 2a).

Hence during a short time-period before the maximum cross-linking has developed, it is possible to detect the formation of 20 kb, 10 kb DNA and of Okazaki-fragments. The present and previous results argue that the reason why we do not detect a 20 kb DNA in undisturbed cells is the rapid and probably simultaneous filling of the gaps present in a replicon. Since the joining of the 10 kb DNA replication intermediates

Fig. 2

(a) Melanoma cells were treated with melphalan (1 ug/ml) for 30 min and then pulsed with tritiated thymidine for 5 min in the absence of melphalan. The cells were lysed in dilute alkali, the solution neutralized and the labelled DNA analyzed by 0.75% agarose gel electrophoresis. 25, 10 and 2 denotes the size (in kb) and location of single-stranded DNA markers. Control cells, cultivated in parallel, were only pulsed with tritiated thymidine for 5 min. -●- melphalan-treated cells, -o- control cells.
(b) Cells treated with melphalan for 30 min were cultivated in fresh medium for further 30 min (-●-) or 90 min (-o-) before labelling with tritiated thymidine and the subsequent cell lysis.
(c) Cells treated with melphalan for 30 min were cultivated in fresh medium for further 4h (-●-) or 24h (-o-) before labelling with tritiated thymidine and cell lysis.
(d) Cells treated with aphidicolin (10 ug/ml) for 90 min and during the last 30 min also with melphalan. The cells were then pulsed with tritiated thymidine for 5 min in the presence of aphidicolin but in the absence of melphalan and were then finally lysed in dilute alkali.

occurs synchronously it is not possible to detect larger DNA replication intermediates. In contrast, when filling of the gaps is not synchronous, as in cells treated with various drugs, it is possible to detect a DNA population consisting of two joined 10 kb DNA molecules.

Taylor has recently proposed a model for DNA replication in mammalian cells in which the replicons are composed of many subunits and each subunit has its potential origin (3). Replication proceeds bidirectionally from the center of the replicon pausing at the potential initiation points when passing the different subunits. According to autoradiographic data the distance between different initiation points is about 12 kb (8), which could possibly correlate to the 10 kb DNA population we have described.

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