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IDENTIFICATION OF THE SHORT DISPERSED REPETITIVE DNA SEQUENCES ISOLATED FROM THE ZONES OF INITIATION OF DNA SYNTHESIS IN HUMAN CELLS AS ALU-ELEMENTS

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SUMMARY: DNA of Xeroderma pigmentosum cells was crosslinked in vivo with trioxsalen and long wave length ultraviolet light and the cells were cultured in the presence of labelled thymidine for one hour. The nascent DNA chains synthesized during this period and containing the DNA replication origins were isolated from the high molecular weight chromosomal DNA by an alkaline sucrose density gradient centrifugation. They were 5-10-fold enriched in short dispersed repetitive sequences identified by dot-blot hybridization to BLUR 8 plasmid as members of the human Alu-family.

Initiation of eukaryotic DNA synthesis occurs at multiple sites scattered along eukaryotic chromosomes and called "replication origins". Although recently some data were reported shedding light on the primary structure of the yeast ARS elements, which are believed to serve as chromosomal replication origins (1, 2), practically nothing is known so far about the size, structure and organization of the replication origins of the higher eukaryotic cells (3). In a previous paper we described a general method for isolation of nascent DNA chains containing the sites

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Abbreviations: Alu, a family of human short repetitive DNA sequences sharing a common Alu I restriction endonuclease site; ARS, autonomously replicating sequences; bp, base pairs; Cot, product of deoxyribonucleotide concentration (moles/l) and time (s); PBS, 140 mM NaCl, 10 mM phosphate buffer, pH 7; SSC, 150 mM NaCl, 15 mM sodium citrate, pH 7; XP, Xeroderma pigmentosum.

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of initiation of eukaryotic DNA replication (4). In this communication we present evidence that the nascent chains isolated from human cells are specifically enriched in Alu-sequences.

MATERIALS AND METHODS

Cells, crosslinking and labelling. Human fibroblasts F-1000 and XP cell line 12BE were grown as monolayer cultures in plastic flascs in Eagle's and Dulbecco's essential media, respectively, supplemented with 20% fetal calf serum. At mid log phase cells were treated 4 times with trioxsalen and near ultraviolet light to crosslink DNA in vivo. To achieve this medium was replaced with PBS containing 2.5 μg/ml trioxsalen (Sigma), samples were kept in the dark for 2-3 min under occasional shaking and were irradiated with a Puva 200 (Waldsmann) lamp containing 15 low pressure mercury tubes 20 W each with an emition maximum at 370 nm. The total flux was 1500 $erg.mm^{-2}.sec^{-1}$ and the irradiation time was 2 min. After the last trioxsalen treatment PBS was replaced with medium containing 25 μCi/ml of [^{3}H] thymidine (75-100 Ci/mmol, NEN) and the trioxsalen treated cells were cultured for 1 hour.

Isolation of DNA. In order to obtain the high molecular weight chromosomal DNA cells were lysed in 1M NaCl, 50mM Tris-HCl, 1% Na-dodecylsulfate, pH 7 at 65°C and were digested with 200 μg/ml of Proteinase K (Merck) at 37°C for 1-2 hours. DNA was deproteinized with chlorophorm-phenol (1:1) and with chlorophorm and was recovered by spooling on a glass rod after adding 1 vol of ethanol. It was denatured in 0.3M NaOH and centrifuged through linear sucrose density gradients prepared in 0.3M NaOH, 1mM EDTA at 25000 rev/min, 10°C for 18 hours in the Beckman SW 27 rotor (5, 6). Gradients were fractionated and aliquots of the fractions were diluted, read at 260nm and the radioactivity was counted. The fractions containing the peak of the nascent DNA chains were pooled together and precipitated with 2 vol of ethanol. DNA was heat denatured and allowed to reassociate in 0.1M NaCl, 5mM Tris-HCl, pH 7.5 at 65°C either in the presence or in the absence of sonicated human DNA as driver. To follow the kinetics of reassociation aliquots were withdrawn at different times and the percentage of double strand material was determined after digestion with S_1 nuclease (4). Double strand DNA from the nascent chains reannealing in the absence of driver DNA was recovered after S_1 nuclease digestion by precipitation with ethanol.

Electrophoresis. Electrophoresis was done in 1% agarose slab gel prepared and run in 40mM Tris-acetate buffer, pH 7.8, 2mM EDTA. Denaturing of DNA in glyoxal-dimethylsulfoxide mixure and electrophoresis of the denatured DNA was carried out as described in (7).

Hybridization. Dot-blot hybridization assay was carried out as described in (8). The double strand DNA isolated from the nascent chains as well as the control DNA were labelled by nick-translation (9) using [^{32}P] dCTP (2000 Ci/mmol, Amersham). Nitrocellulose filters were autoradiographed by exposure to X-ray (Orwo) film in the presence of an intensifying screen at -75°C.

RESULTS AND DISCUSSION

In order to isolate human DNA replication origins we applied exactly the procedure described earlier for mouse tumor cells (4).

Briefly, cellular DNA was crosslinked in vivo by several succesive treatments with trioxsalen and near ultraviolet light and the cells were then cultured in the presence of radioactive precursor for one hour. Crosslinking did not greatly affect the initiation of the DNA synthesis but efficiently blocked the elongation of the initiated nascent DNA chains which led to the synthesis and accumulation of short DNA fragments containing the regions of initiation of DNA synthesis. These fragments were dissociated from bulk DNA under denaturing conditions and isolated by sucrose density gradient centrifugation (4, 5). Two human cell lines - F-1000 fibroblasts and XP 12BE were used in these experiments. F-1000 exhibited normal repair synthetic ability, while the excision repair in XP cells was 2.5%. The two cell lines were crosslinked by four succesive trioxsalen treatments and the cross-link concentration (determined as in (4)) was $7x10^{-4}$ - 10^{-3} cross-links per bp which gave an average distance of 1 - 1.5 kb between neighbouring crosslinks. In order to detrmine the amount and size distribution of the nascent DNA fragments synthesized between crosslinks in the two cell lines, total chromosomal DNAs were denatured and electrophoresed. In the case of F-1000 fibroblasts the bulk of DNA remained at and near the sample slot. This DNA contained about 60% of the total radioactivity incorporated as a result of repair synthesis. The remaining radioactivity was incorporated into a negligable amount of DNA and migrated as a well shaped peak of 1 -1.2 kb (Fig. 1). It represented the population of the nascent DNA chains synthesized between crosslinks. When these experiments were carried out with XP cells, generally the same picture was obtained (Fig. 1) with the only difference that the radioactivity incorporated in the high molecular DNA peak was considerably lower due to the reduced repair synthetic ability of these cells (10). The fact that in the two cell lines the in-

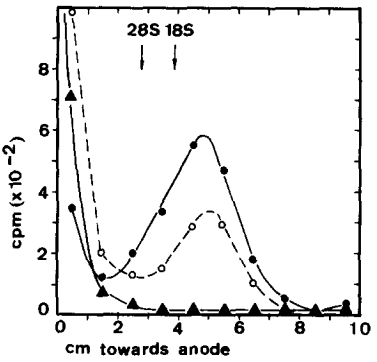

Fig. 1 Electrophoresis of F-1000 and XP crosslinked DNA under denaturing conditions.
Cells were treated with trioxsalen and near ultraviolet light and labelled with [^{3}H] thymidine for 1 hour as described in text. The crosslinked DNA was isolated, denatured in glyoxal-dimethyl-sulfoxide mixture (7) and electrophoresed in 1% agarose gel. Gel was cut into 1 cm strips, solubilized and counted as described in (4). Denatured rat ribosomal 18S and 28S RNAs were run in parallel as molecular weight markers.
o----o, counts of F-1000 DNA; ●——●, counts of XP DNA; ▲——▲, ethydium bromide staining (arbitrary units).

corporation in the lighter DNA peak was approximately the same regardless of their very different excision repair capacity was an additional indication that this DNA fraction was a product of semiconservative DNA synthesis initiated between crosslinks and not of repair synthesis. XP cells were used in the further experiments since in this case repair would not greatly interfere with the semiconservative replication taking place between cross-links. The crosslinked DNA was denatured in 0.3M NaOH and resolved by centrifugation through alkaline sucrose density gradients. The peak of the nascent DNA was collected and DNA was isolated. Its specific radioactivity was about 50 times that of the bulk DNA which was interpreted that it was highly enriched in newly synthesized DNA fragments. This DNA was additionally heat denatured and allowed to reassociate in the absence of any driver DNA to Cot 5. Single strand DNA was digested with S_1 nuclease and the double strand DNA, which represented 25-30% of the total fraction isolated from the gradient was recovered. The electrophoretic

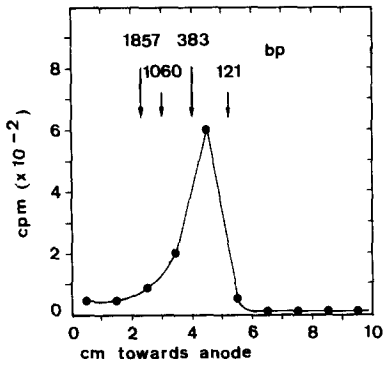

Fig. 2 Electrophoresis of the repetitive DNA isolated from the nascent chains synthesized between crosslinks.
XP cells were crosslinked and labelled as described in Fig. 1, DNA was denatured and centrifuged through 5-20% alkaline sucrose density gradient. The nascent DNA fraction was recovered from the corresponding zones of the gradient, allowed to renature in the absence of any driver DNA to Cot5, the single strand DNA was digested with S_1 nuclease and the double strand DNA was electrophoresed in 1% agarose gel. Gel was cut and counted as in (4). Mbo I digest of pBR 322 was electrophoresed in parallel to calibrate the gel.

analysis showed that it migrated as a rather homogeneous peak of approximately 300 bp (Fig. 2). Reassociation kinetics of this DNA fraction to an excess of sonicated total human DNA revealed that it represented sequences repeated 2-5x10^5 times per haploid genome (not shown). Both size and copy number of these fragments indicated that they might represent Alu-sequences (11, 12). To check this point we immobilized in duplicate on nitrocellulose filters different amounts of BLUR 8, a plasmid containing a cloned human Alu- sequence. One set of filters was hybridized to in vitro labelled by nick-translation double strand DNA fraction isolated after renaturation and S_1 nuclease digestion of the nascent chains (Fig. 3A). The second set of filters was hybridized to in vitro labelled to same specific radioactivity control DNA obtained by renaturation of denatured total human DNA to Cot 5 and digestion with S_1 nuclease (Fig. 3B). The comparison of the autoradiographs of the filters revealed that the DNA fraction isolated from the nascent chains was 5-10-fold enriched in Alu-

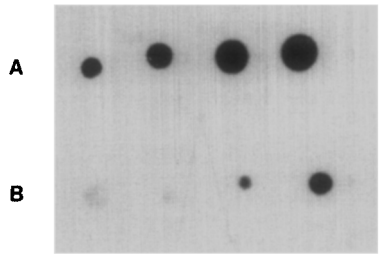

Fig. 3 Hybridization of repetitive DNA isolated from the nascent chains (A) and of control repetitive DNA (B) to BLUR 8. 10, 20, 50 and 200 ng of BLUR 8 were denatured and applied in duplicate to nitrocellulose filters as described in (8). One set of filters (A) was hybridized with in vitro labelled double strand DNA isolated from the nascent chains as described in Fig. 2. In a parallel experiment (B) the second set of filters was hybridized with in vitro labelled to the same specific radioactivity control double strand human DNA prepared by reassociation of heat denaturated total DNA to Cot 5 followed by digestion with S_1 nuclease. Filters were washed in 0.1 x SSC, 0.5% SDS at 68°C for 1 hour and exposed to X-ray film.

sequences (Fig. 3). The fact that Alu-sequences were specifically present in the zones of initiation of human DNA synthesis confirmed the suggestion of Jelineck et al (12) that the Alu-sequences might play a role in the process of initiation of DNA synthesis in vivo.

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