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Replicon map of the human dystrophin gene: asymmetric replicons and putative replication barriers

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Summary

Using the replication direction assay and oligonucleotide probes designed on the basis of the known exon sequences of the human *dystrophin* gene we have made a replicon map of this giant gene. It has been found that *dystrophin* gene is organized into at least six replicons ranging in size from 170 to more than 500 kb. One of the replicon junctions (sites of replication termination) was mapped in intron 44, i.e. roughly in the same area where the major recombination hot spot is located. It is also worth mentioning that the central part of the *dystrophin* gene (exons 8 - 48) is organized into relatively short symmetrical replicons surrounded by two extended regions of apparently unidirectional replication (exons 1 - 8 and exons 49 - 64). These observations suggest for the first time that there should be certain signals for the termination of replication in euchromatic areas of the genome of higher eukaryotes. Furthermore, it may be concluded that the replication of the central part of *dystrophin* gene must be completed much faster than the replication of its ends. This may induce some topological stresses resulting in an increased rate of chromosomal rearrangements within this gene. The experimental approach used in our study may be helpful for fast analysis of the replication structure of other areas of the human genome provided that these areas are saturated with STS markers.

I. Introduction

The human dystrophin gene is the largest gene so far identified and characterized. It extends over 2 mb on the short arm of the X-chromosome (Burmeister et al., 1988). This gene frequently undergoes different rearrangements causing Duchenne or Becker muscular dystrophy (Wapenaar et al., 1988; Den Dunnen et al., 1989; Blonden et al., 1991). Analysis of the replication structure of the dystrophin gene may give new insight into the mechanisms of this gene rearrangement as it seems probable that at least some recombination events occur in connection with DNA replication.

It has long been shown that the genome of higher eukaryotes is replicated as a set of quazi-independent replication units (replicons). Each replicon seems to possess a specific site (or area) where the replication starts (for a review see Hamlin, 1992; DePamphilis, 1993; Hamlin and Dijkwel, 1995). As far as the sites of termination of DNA replication (i.e. replicon junctions) are concerned, the situation seems to be less clear. Although these sites can be mapped using the analysis of replication polarity (see below and also Handeli et al., 1989), it is possible that their positions are determined simply by a distance from the replication origins and by the speed of replication forks progression. Such is indeed the case in the simian virus 40 circular genome, as the insertion in one arm of the SV-40 replicon of a DNA sequence element retarding the progression of the replication fork was found to cause a displacement of the replication termination site in the direction of the more slowly moving replication fork (Rao et al., 1988; Rao, 1994). In yeast cells the termination of replication does not occur at specific places determined (at least in non-nucleolar regions) by any specific DNA sequence element. It appears to be a consequence of converging of the replicating forks within a relatively broad region (Zhu et al., 1992). At the same time, some DNA sequences pausing the replication forks progression (such as the transcription termination signal for RNA polymerase I) were reported to serve as preferential sites of replication

termination in yeast and mammalian cells (Umek et al., 1989; Kobayashi et al., 1992; Little et al., 1993).

One may be surprised to realise how little we know about replication structure of DNA of higher eukaryotes. Even the average size of replicons constitutes a matter of discussion. The common view is based on the results of DNA fiber radioautography studies carried out more then 20 years ago. These studies lead to a conclusion that DNA of higher eukaryotes is organized in clusters of simultaneously working replicons. The size of individual replicons within a cluster was estimated as 50 to 300 kb (Huberman and Riggs, 1966, 1968; Callan, 1974; Stubblefield, 1974; Edenberg and Huberman 1975; Painter, 1976). This common interpretation of the DNA fiber radioautography data was, however, questioned by Liapunova and coauthors who presented arguments for the much larger size of replicons (150-900 kb) in mammalian cells and for the absence of replicon clusters (Yurov Yu. B. and Liapunova, 1977; Liapunova, 1994). Several procedures for mapping replication origins in mammalian genome have been developed recently (for review see Hamlin, 1992; Vassilev and DePamhilis, 1992; DePamphilis, 1993; Hamlin and Dijkwel, 1995). However, most of these procedures are not suitable for the analyzis of replication structure of large genomic areas. Only one modern protocol, namely that based on the determination of the polarity of leading DNA strand synthesis (Handeli et al., 1989; Burhans et al., 1991) may be used for this purpose as it is relatively simple and permits the approximate positions of both replication origins and termination sites to be mapped.

Here we are presenting a replicon map of the dystrophin gene constructed using the replication direction assay. It has been found that this gene is organized into at least six replicons ranging in size from 170 to more than 500 kb. One of the replicon junctions (sites of replication termination) was mapped in intron 44, i.e. roughly in the same area where the major recombination hot spot is located (Wapenaar et al., 1988; Den Dunnen et al., 1989; Blonden et al., 1991). The experimental approach used in our study (utilization of oligonucleotide probes in the replication direction assay) may be helpful for fast analysis of the replication structure of other areas of the human genome provided that these areas are saturated with STS markers.

Figure 1. A scheme illustrating the experimental procedure used to determine the polarity of leading DNA strand synthesis. The nascent DNA chains in a replication loop are shown by thick arrows. Short arrows show ligated Okazaki fragments (synthesised before addition of emetine). The scheme is based on the data of Burhans et all. (1991) who have demonstrated that emetine induce imballanced DNA synthesis. Although based on a wrong assumption, the protocol for determining the polarity of leading DNA synthesis was developed two years earlier by Handeli et al. (1989).

II. Results

A. Mapping approach

Determination of the polarity of leading DNA strands synthesis became possible due to the demonstration that the inhibition of protein synthesis in proliferating cells preferentially suppresses the synthesis of the discontinuous (lagging) DNA strand. Hybridization of the nascent DNA synthesised under these condition with strand-specific probes can thus be used to assay the polarity of leading DNA strand synthesis (Handely et al., 1989; Burhans et al., 1991). The principle of the above-described mapping protocol is illustrated in **Fig. 1**. Although the mechanism of imbalanced synthesis of leading and lagging DNA strands in the presence of protein synthesis inhibitors is still not known, the validity of the approach has been verified in experiments with different genomic areas (Handely et al., 1989; Burhans et al., 1991; Kitsberg et al., 1993) and can hardly be questioned. It was originally proposed to use as strand-specific probes for the replication direction assay the RNA chains transcribed in opposite directions from the same DNA fragment (Handely et al., 1989). Naturally these probes could be made only after cloning of the necessary DNA fragment in an appropriate vector. In order to facilitate the mapping protocol we have developed conditions for using 20-mer oligonucleotides as strand-specific probes. To test the approach we have analysed the direction of replication forks movement within the domain of chicken alpha-globin genes (Verbovaia and Razin, 1995). The results obtained were

in perfect agreement with the previously published data on mapping the replication origin in this domain. Oligonucleotide probes can be easily washed out from the filters and the same filters with immobilized nascent and total DNA from cells treated with emetine or other inhibitor of protein synthesis can be used sequentially in a number of hybridization experiments. To study the replication structure of human dystrophin gene we have used HEL 92.1.7 cells derived from a male patient as it was not clear whether the replication structures of the active and non-active copies of the X-chromosome in female cells were identical. The cells were cultivated for 18 h in presence of emetine and 5-bromo-2'-deoxy-uridine (BrdU) exactly as described by Handeli et al. (1989). (See also Methods section in the end of this paper). The DNA was then isolated, denatured, sheared to about 1 kb fragments and nascent DNA chains containing BrdU were separated from the bulk DNA by double immunoprecipitation, as described previously (Vassilev and Russev, 1988). Equal amounts (2 μg) of total DNA and nascent DNA from emetine-treated cells were immobilised on nylon filters and hybridized with oligonucleotide probes representing complementary DNA chains.

In order to exclude the possibility of artefacts due to the uneven sorption of DNA on filters, each filter was sequentially hybridized to probes derived from both strands and each pair of probes was hybridized to at least two different filters. In all cases the results of these four hybridization experiments confirmed each other. A typical example is shown in **Fig. 2**. Two similarly prepared filters with immobilized nascent and total DNA were hybridized to the "lower chain" and the "upper chain" probes derived from the sequence of the brain promoter of the dystrophin gene (here and further we use

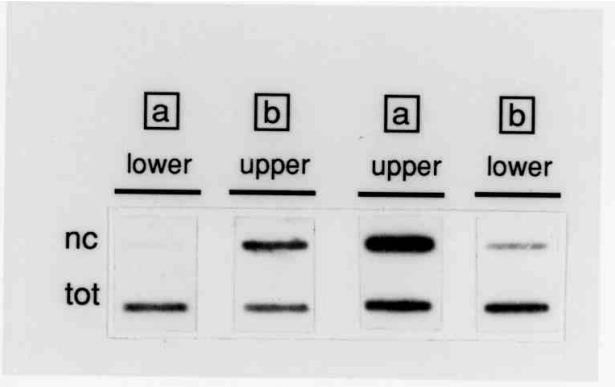

Figure 2. Reciprocal hybridization of "lower chain" and "upper chain" oligonucleotide probes from the dystrophin gene brain promoter with nascent (nc) and total (tot) DNA immobilized on two similarly prepared filters. The filter "a" was first hybridized to the "lower chain" probe and then, after exposure and dehybridization, to the "upper chain" probe. The filter "b" was first hybridized to the "upper chain" probe and then, after exposure and dehybridization, to the "lower chain" probe. Note the preferential hybridization of the nascent DNA with the "upper chain" probe in both cases.

the designation "upper chain" for the chain which is transcribed into dystrophin pre-mRNA). This experiment has demonstrated preferential hybridization of the "upper chain" probe to the nascent DNA. After exposure, the probes were washed off the filters and the "upper chain" probe was hybridized to the filter previously hybridized to the "lover chain" probe and vise versa. Again, preferential hybridization of the "upper chain" probe with the nascent DNA was observed. The asymmetry of hybridization of the "lower chain" and the "upper chain" probes to the nascent DNA remained visible even after high-stringency wash (wash with 0.1X SSC-0.1%SDS for 15 min at 42^0C instead of normally used wash with 1X SSC for 15 min at 42^0C).

B. Mapping of replication units within the dystrophin gene

To assay the polarity of replication of different parts of the dystrophin gene we prepared 36 pairs of oligonucleotide probes (**Table I**). Some of these probes were made on the basis of the previously described primers for STS markers (Coffey, et al., 1992). These probes are referred to by their name in the original publication (Coffey, et al., 1992) with the number of a corresponding exon indicated in parentheses. Other oligonucleotide probes were designed on the basis of the known primary structure of dystrophin mRNA (Koenig et al., 1987) and the exon-intron structure of the dystrophin gene (Roberts et al., 1993). These probes are referred to by the number of a corresponding exon. Approximate positions of the probes on the physical map of the dystrophin gene are shown in **Fig. 3A**.

The results of hybridization of the whole set of strand-specific probes with total DNA and nascent DNA samples enriched in leading strands are shown in **Fig. 3 B**. The polarity of the leading DNA strand synthesis was found to switch eleven times within the area under study. Keeping in mind the fact that the replication forks meet at the termination sites and move in opposite directions from the replication origins one can say that the area under study contains 5 replication origins and 6 termination sites. The first of the termination sites is located between the brain and muscle promoters. Indeed, the brain promoter (R24 probes) is replicated in the direction of dystrophin gene transcription, while the muscle promoter (R22(E1) probes) and exons 2 to 7 (probes R12(E2), R13(E3) and R7(E7)) are replicated in the direction opposite to the direction of transcription. This conclusion follows from preferential hybridization of the nascent DNA leading strands with the "upper chain" probe of the R24 pair and with the "lower chain" probes of the R22(E1), R12(E2), R13(E3) and R7(E7) pairs, as shown schematically in **Fig. 4**. The next switch in replication polarity occurs between exons 7 and 8. This is a switch from the minus chain to the plus chain which is indicative of the presence of a replication origin between probes R7(E7) and R2(E8) (see the scheme in **Fig.** 4). Similar considerations make it possible to

conclude that the replication origins are located between exons 28 and 29, between exons 43 and 44, between exons 46 and 48 and between exons 64 and 68. The replication termination sites are located between probes 87-1 and 87-15, between exons 40 and 43, between exons 44 and 45, between exons 48 and 49 and between exons 70 and 75.

Table I. Oligonucleotide probes used for determination of the dystrophin gene replication structure.

Names of probes	Nucleotide sequence of the probe from the "upper" chain	Nucleotide sequence of the probe from the lower chain
R24	CTTTCAGGAAGATGACAGAATC	GATTCTGTCATCTTCCTGAAAG
R22(E1)	CTTTCCCCCTACAGGACTCAG	CTGAGTCCTGTAGGGGGAAAG
R12(E2)	GAAAGAGAAGATGTTCAAAAG	CTTTTGAACATCTTCTCTTTC
R13(E3)	GGCAAGCAGCATATTGAGAAC	GTTCTCAATATGCTGCTTGCC
R7(E7)	CTATTTGACTGGAATAGTGTG	CACACTATTCCAGTCAAATAG
R2(E8)	CCTATCCAGATAAGAAGTCC	GGACTTCTTATCTGGATAGG
R14(E11)	GTACATGATGGATTTGACAGC	GCTGTCAAATCCATCATGTAC
87-1	CTATCATGCCTTTGACATTCCA	TGGAATGTCAAAGGCATGATAG
87-15	ATAATTCTGAATAGTCACA	TGTGACTATTCAGAATTAT
R21(E25)	CAATTCAGCCCAGTCTAAAC	GTTTAGACTGGGCTGAATTG
R25(E27)	GCTAAAGAAGAGGCCCAAC	GTTGGGCCTCTTCTTTAGC
E28	GTTTGGGCATGTTGGCATGAG	CTCATGCCAACATGCCCAAAC
E29	TGCGACATTCAGAGGATAACC	GGTTATCCTCTGAATGTCGCA
E31	GGCTGCCCAAAGAGTCCTGTC	GACAGGACTCTTTGGGCAGCC
R16(E33)	GTCTGAGTGAAGTGAAGTCTG	CAGACTTCACTTCACTCAGAC
E35	GAAGGAGACGTTGGTGGAAGA	TCTTCCACCAACGTCTCCTTC
R31(E39)	CAACTTACAACAAAGAATCACA	TGTGATTCTTTGTTGTAAGTTG
R8(E40)	GGTATCAGTACAAGAGGCAG	CTGCCTCTTGTACTGATACC
E43	GTCTACAACAAAGCTCAGGTCG	CGACCTGAGCTTTGTTGTAGAC
E44	GACAGATCTGTTGAGAATTGC	GCATTTCTCAACAGATCTGTC
R18(E45)	CTCCAGGATGGCATTGGCAG	CTGCCAATGCCATCCTGGAG
R4(E46)	ATTTGTTTTATGGTTGGAGG	CCTCCAACCATAAAACAAAT
E48	GTTTCCAGAGCTTTACCTGA	TCAGGTAAAGCTCTGGAAAC
E49	ACTGAAATAGCAGTTCAAGC	GCTTGAACTGCTATTTCAGT
E50	GAAGTTAGAAGATCTGAGCTC	GAGCTCAGATCTTCTAACTTC
E53	CAGAATCAGTGGGATGAAGTA	TACTTCATCCCACTGATTCTG
E54	CCAGTGGCAGACAAATGTAG	CTACATTTGTCTGCCACTGG
E55	TGAGCGAGAGGCTGCTTTGG	CCAAAGCAGCCTCTCGCTCA
R20(E56)	GGTGAAATTGAAGCTCACAC	GTGTGAGCTTCAATTTCACC
E60	ACTTCGAGGAGAAATTGCGC	GCGCAATTTCTCCTCGAAGT
E61	GCCGTCGAGGACCGAGTCAG	CTGACTCGGTCCTCGACGGC
E64	ACTCCGAAGACTGCAGAAGG	CCTTCTGCAGTCTTCGGAGT
E68	TAAGCCAGAGATTGAAGCGG	CCGCTTCGATCTCTGGCTTA
E70	ACATCAGGAGAAGATGTTCG	CGAACATCTTCTCCTGATGT
E75	CTGCAAGCAGAATATGACCG	CGGTCATATTCTGCTTGCAG
R5(E79)	CAGAGTGAGTAATCGGTTGG	CCAACCGATTACTCACTCTG

Figure 3 (Following page). Determining replication polarity within the dystrophin gene. (A) A scheme illustrating the exon-intron structure of the dystrophin gene and the results of determination of replication polarity. On the map of the dystrophin gene the exons are shown by vertical dark bars. Each tenth exon is indicated by the number. Positions of the brain and muscle promoters are shown by arrows above the map. The results of the analysis of replication direction are shown below the map. The vertical bars indicate the positions of the probe pairs used to assay the replication polarity. The direction of replication determined by hybridization of nascent DNA with each of the probe pairs is shown by horizontal arrows. Approximate positions of the origins (ori) and termination sites (t) are indicated above the arrows. (B) Hybridization of strand-specific probes with total DNA (tot) and nascent DNA (nc) from emetine-treated cells. The names of the probe pairs are indicated above the autoradiographs. "-" and "+" indicate the results of hybridization with probes derived from the lower and the upper chains, respectively.

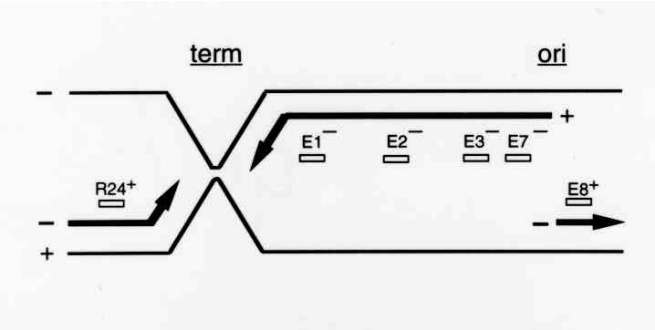

Figure 4. A scheme illustrating the interpretation of the results of hybridization of strand-specific probes with DNA samples enriched in nascent DNA leading strands. The upper chain and the lower chain probes are designated correspondingly by "+" and "-".

III. Discussion

A. The size of replicons

The present study has demonstrated for the first time that a single gene may be organized into several replicons. The average size of replicons mapped within the area under study constitutes 500 kb (with variations from 170 to 1000 kb). This finding contradicts to the common view that the average sizes of replicons in mammalian cells are from 50 to 300 kb. However our observations are in perfect agreement with the estimations of replicon sizes made by Liapunova and Yurov (reviewed by Liapunova, 1994). Furthermore, analysis of the temporal order of DNA replication in the H-2 mouse majour histocompactibility complex also suggested that mammalian replicons are larger then 300 kb (Spack et al., 1992). Similar conclusion follows from the results published by Bickmore and Oghene (1996).

B. Asymmetrical replicons and replication barriers.

The results of the present study demonstrate that in the human genome the replicons may be asymmetrical. Indeed, an extended (500 kb) region including exons 49 - 64 seems to be replicated unidirectionally. The opposite arm of the same replicon is relatively small (less than 100 kb). It is possible that the left end of the dystrophin gene (500 kb DNA stretch) is also replicated unidirectionally. At least all exons scattered along this region are replicated in the same direction. Some of the replication termination sites mapped in the present study are not located at the middle of the distance between two neighbouring origins. This suggests that there should be some specific signals determining positions of termination sites. Up to now the replication barriers of this kind were observed only in yeast and mammalian ribosomal genes clusters (Umek et al., 1989; Kobayashi et al., 1992; Little et al., 1993).

C. The replication structure of the dystrophin gene and recombination hot-spots

It may be of interest that one of the replication junctions (termination sites) identified in the present study is located in intron 44, i. e. roughly colocalizes with the main recombination hot-spot in the dystrophin gene (Wapenaar et al., 1988; Den Dunnen et al., 1989; Blonden et al., 1991). Although the significance of this colocalization (if any) is not presently clear, it is worth mentioning that in prokaryotic cells the sites of replication termination have long been known to constitute recombination hotspots (Bierne *et al*., 1991; Horiuchi *et al.*, 1994; Horiuchi *et al*., 1995). According to one of the models, the replication fork posed at a termination site is a weak point on DNA where a double-stranded-break may occur with a high probability (Horiuchi *et al*., 1995; Michel et al., 1997). Some data suggest that a similar mechanism may account for the formation of recombination hot-spots also in eukaryotic cells (Horiuchi *et al.*, 1995). In agreement with this idea it was demonstrated that pausing of the replication machinery by certain DNA secondary structures, DNA damage or DNA-protein interaction cause an increase in the rate of DNA rearrangements (Bierne and Michel, 1994). It is known that in eukaryotic cells finalization of DNA replication (juncture of neighbouring replicons) is a relatively slow process. During this step the replication forks retain single-stranded regions which can be relatively easy converted into double-stranded breaks. Furthermore, merging of replicons depends on the reactions catalysed by DNA topoisomerases which seem to be able under certain conditions to carry out illegitimate recombination of DNA strands and hence to introduce deletions and insertions into DNA (Gale and Osheroff, 1992; Shibuya et al., 1994; Henningfeld and Hecht, 1995; Bierne et al., 1997).

An interesting feature of the replication structure of dystrophin gene is that the central part of the gene (exons 8 - 48) is organized into relatively short symmetrical replicons which are surrounded by two extended regions of apparently unidirectional replication (exons 1 - 8 and exons 49 - 64). Assuming that the rate of replication forks progression is the same in all replicons, it may be concluded that the replication of the central part of the gene must be completed much faster than the replication of its ends. This may cause some topological stresses resulting in an increased rate of chromosomal rearrangements within the dystrophin gene.

IV. Methods

A. Cell culture.

Human erythroleukemia cells HEL 92.1.7 were purchased from the American Type Culture Collection. The cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum.

B. Isolation of DNA samples enriched in nascent DNA leading strands.

To induce imbalanced synthesis of nascent DNA strands, exponentially growing cells were treated with emetine, as described previously (Handeli et al., 1989; Burhans et al., 1991). Emetine was added to the conditional medium up to a concentration of 2 μM. This was followed (after 15 min incubation) by the addition of 5-bromo-2'-deoxy-uridine (10 μg/ml) and ^{3}H deoxy-cytidine (2 μCi/ml). The cells were cultured in this medium for 16 h. Then they were collected and their DNA was isolated. After shearing (to give fragments with an average size of 1 kb) and denaturation of the DNA, the BrdU-labelled nascent DNA chains were separated from the bulk DNA by double immunoprecipitation, as described previously (Vassilev and Russev, 1988).

C. Immobilization of DNA on nylon filters and hybridization experiments.

Equal amounts (2 μg) of the nascent and bulk DNA were immobilized on Hybond-N+ nylon filters (Amersham) using a Bio-Dot SF microfiltration unit (Bio-Rad). The equivalency of immobilization of all probes was verified by hybridization with ^{32}P-labelled human repeated sequence of alu type. The oligonucleotides were labelled with γ^{32}P-ATP using T4 phage polynucleotide kinase, as described previously (Maniatis et al., 1982). Hybridization was carried out in a Rapid Hyb solution (Amersham) for 1 h at 42 ^{0}C. After hybridization, the filters were washed one time in 5XSSC - 0.1% (w/v) SDS solution for 20 min at room temperature and two times (15 min each) in 1X SSC - 0.1% (w/v) SDS solution at 42 ^{0}C. Then the filters were exposed to the Kodak film at -75 ^{0}C with an intensifying screen (Dupont). For dehybridization of the radioactive probes the filters were incubated in 0.4 M NaOH solution for 30 min at 45 ^{0}C. Then they were neutralized (15 min at room temperature) in the following solution: 0.1X SSC - 0.1%(w/v)SDS - 0.2M Tris-HCl (pH 7.5).

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