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An ARS element from *Drosophila melanogaster* telomeres contains the yeast ARS core and bent replication enhancer

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ABSTRACT

We have sequenced the 0.7-kb-long fragment of Drosophila DNA which ensures the autonomous replication of plasmids in yeast. Deletion mapping has shown the ARS element to consist of at least two domains: the core having the consensus 11-bp sequence TAAATATAAAT and the enhancer which is no more than 90 bp long and is located at the 3'-end of the A-rich core strand. Neither domaine per se ensures plasmid replication in yeast. A comparison of the enhancer sequence with the sequences of 14 different ARS elements failed to reveal significant homology areas. Most probably the ARS flanks that are adjacent to the core and act as enhancer do not carry any consensus. They may determine a peculiar structural feature of DNA (for example bends) which are necessary for the protein - ARS interaction.

INTRODUCTION

Sequences ensuring the autonomous replication of plasmids in Saccharomyces cerevisiae cells (ARS elements) have been revealed in the DNA of various eukaryotic organisms (1). ARS are AT-rich sequences of no less than several dozen base pairs without extensive regions of mutual homology (2). All the ARS elements exhibit an 11-bp consensus, (A/T)TTTATPuTTT(A/T), which is necessary but not sufficient for effective replication (2,3,4). Having studied various deletion derivatives of the ARS elements, different researchers arrive to the conclusion that, besides the core, additional enhancer sequences are needed (5-10) to raise the effitiency of replication. Authentic yeast ARS sequences have properties expected of origins of chromosomal replication (1).

We have earlier cloned a fragment of Drosophila DNA, Dm665, which hybridizes in situ with the telomeres of polytene chromosomes and contains an ARS element (11). In the present

study we localize the ARS sequence within this fragment and show that the 90-bp-long segment at the 3'-end of the A-rich core strand is essential to replication. This enhancer per se does not ensure plasmid replication in yeast, but in combination with AT-blocks that are similar in structure to the consensus core it creates a functional ARS. A comparison of the enhancer with other ARS elements failed to reveal any significant regions of homology. We also failed to find in our sequence the enhancer consensus reported by others (12). We believe that the enhancer sequences adjacent to the core have no conserved regions identifiable as consensus. Possibly they create some kind of DNA or chromatin structure that favours replication.

MATERIALS AND METHODS

Strains and media. E.coli strains LE392 and JM83 were used for cloning. Transformants were grown on an LB medium (13) with 100 μg/ml ampicillin. The transformation of the yeast strain DBY747: a, his3-1, leu2-3, leu2-113, ura3-52, trp1-289 was carried out in the presence of lithium chloride (14). The transformants were grown on a selective medium containing 0.67% Yeast Nitrogen Base w/o amino acids (DIFCO), 2% glucose and the necessary amino acids in a concentration of 20 μg/ml. The growth rate of yeast transformants on the selective medium was determined by measuring the turbidity of the cultures.

Determination of the ARS activity of plasmids. The yeast integrative plasmid YIp5 (15), comprising the pBR322 plasmid and the yeast gene URA3, does not give rise to any transformants when one uses 1-5 μg of DNA per 10^7 yeast cells. The reason for this is the lack of plasmid replication in yeast. The insertion of ARS in the plasmid causes the efficiency of transformation to reach 100-500 per 1-5 μg of DNA as a result of autonomous replication of plasmids.

Deletions in plasmid DNA. Bal31 generated deletions in plasmid DNA were obtained by standard procedure (13).

DNA sequencing was performed according to Maxam and Gilbert (16).

Computer analysis of the DNA sequence was based on the

SEQBUS software package deloped at the Institute of Molecular Genetics, the USSR Academy of Sciences.

RESULTS

The efficiency of Drosophila ARS in yeast.

Earlier we have cloned a DNA fragment from the Drosophila genome, Dm665, which is capable of ensuring the autonomous replication of plasmids in yeast cells (11). Hybridization with polytene chromosomes has demonstrated that the fragment has homology regions in the telomeres of chromosomes X, 2 and 4.

The replication efficiency of the ARS located in Dm665 was assessed by the growth rate of yeast cells carrying the p665-52 plasmid (Fig.1) on a selective medium. The slowing down of cell growth in this situation reflects the frequency of plasmid loss, which is associated with the efficiency of plasmid replication and the accuracy of partition (17). A yeast strain carrying the YRp12 plasmid with the ARS1 yeast replicator was used for control (15). The growth rates of cultures containing plasmids with heterologous and homologous ARS were found to be practically the same (Table 1, lines 2 and 3). Hence the ARS of Dm665 has a high replication efficiency in yeast.

To rule out the effect of unstable inheritance due to incorrect segregation of plasmids in dauther cells, we constructed the plasmid p665-53-CEN3 containing the centromere from chromosome III of yeast. The centromere ensures an accurate distribution of plasmids among the doughter cells and maintains the copy nomber at 1-2 per cell (18). The growth rate of yeast cells with such plasmids under selective conditions reflects the efficiency of the plasmid replicator (4). The yeast plasmid CEN3(41) was used for control (18). Yeast cells carrying this plasmid grow at the same rate as the haploid plasmid-free strain (Table 1, lanes 1 and 4), i.e. the ARS1 ensures effective replication of the plasmid. The introduction of the centromere into the Drosophila ARS-carrying plasmid also considerably enhances the growth rate, even though it never reaches the control level (Table 1, lanes 4 and 5). Thus the Dm665 fragment carries ARS comparable in efficiency with ARS1.

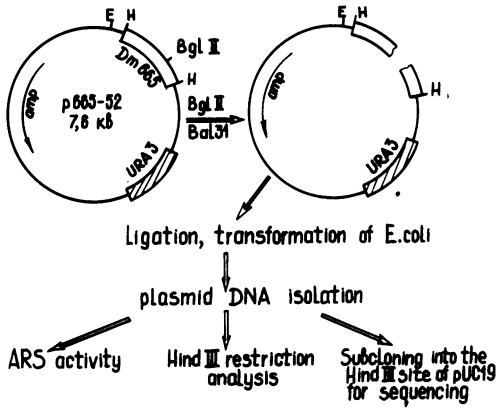

Fig. 1. The procedure for obtaining and analysing deletions in tne Dm665 fragment.
amp - ampicillin resistance, URA3 - marker yeast gene. Restriction sites: E - EcoRI, H - HindIII.

Localization of the ARS in the Dm665 fragment.

The Dm665 fragment (2.4 kb) has been cloned using the HindIII endonuclease. The single internal BglII restriction site divides the fragment into 1.7kb and 0.7kb subfragments (Fig.1). The subcloning of these subfragments in the yeast integrative plasmid YIp5 has shown the ARS to be located in the smaller one (Table 2). For a more precise mapping a set of deletions was obtained by linearizing the p665-52 plasmid with BglII followed by the Bal31 exonuclease treatment. Fig.1 illustrates the procedure for obtaining and analysing the the deletion derivatives.

Table 1. Generation time of plasmid-carrying yeast strains under selective conditions

Strain	Generation time (hours)
1. DBY747	2.5
2. DBY747/YRp12	4.7
3. DBY747/p665-52	4.9
4. DBY747/CEN3(41)	2.3
5. DBY747/p665-52-CEN3	3.4

Table 2. Localization of ARS within the Dm665 fragment by subcloning and deletion analysis

Plasmid	Dm665 Fragment Size (kb)	Deletion Bounderies			Replication in Yeast
		HindIII	BglII	HindIII	
p665-52	2.4	----------			+
p665-53	0.7		-------		+
p665-54	1.7	----------			-
pSL44	1.5	----------		-----	+
pSL41	1.3	----------		-	+
pSL52	1.4	----------		-	+
pSL24	0.9	-----		----	+
pSL4	1.0	----------			-

The plasmids of the p665 series were obtained by subcloning and pSL-plasmids by treatment of the BglII restricted p665-52 with Bal31. Only the representative subset of pSL deletion derivatives is shown.

Each plasmid carrying the deletion was tested for replication in yeast according to transformation efficiency. The results are shown in table 2. All the deletion derivatives keeping both flanks of the Dm665 fragment, i.e. carrying two HindIII restriction sites, display replication activity. None of the derivatives that had lost the smaller BglII-HindIII subfragment (0.7 kb), i.e. had only one HindIII site, showed any replication in yeast. Thus, ARS is located in close proximity to the HindIII end of the 0.7 kb subfragment.

Sequencing Dm665 and its deletion derivatives.

The nucleotide sequence of the ARS-containing 0.7 kb fragment is shown in Fig.2. The 11 bp sequence TAAATATAAAT located at a distance of 120 bp from the HindIII site coincides with the consensus core sequence previously established for various ARSs (2).

For three plasmids with the largest deletions which keep both HindIII sites and display ARS activity, pSL41, pSL52 and

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GATCTNNNNN CCTCGCTATA TCAACGTCTT CGTGCCGCGA TCCAACTGGA  50
CGGAGCCATG TTCGGGACCA CCTTCCCGCA CATGTTCTTG ACATGTTAGC 100
TGCCGAACTT TAGACCCCAG CCGCTCCTCT CTGCTATGCA GGAGTATATG 150
TAAGCCGAGT ATACATGGGT ACATACTTTC CAATACGCCA CCTCAAAAGT 200
TACATTGCTG TACTTTTATT ACTTGCATGT CCTTCTGGGG AATAATTTTG 250
ATCCAGCATT CGCTGCGAGG TGTGGGAAAC CAGGTGGGAA ACCCAGTAAA 300
ACCCAACCAG ATCAATTGAA CATATTATTT ACATAAATTC AGTTTGCAAA 350
CAATTTTAAC TAAAATCAAT ATTATTCAAC TTTGTAGAAC CTCAAAATTA 400
TCAATAAACT TAATTAAATT TAATTTAATG ATACTTTATA TCACGACCCC 450
CAGTTCAGTC TGTTTCTTCA TTTCAATCTA AGCATATATT GTTTTAATTC 500
ATTCTGAGAG TCCAAGCCCA CTTCTCAATG TAATGAATCT TGCAGATGGT 550
AACGGCTTAG TTAGTATATC AGCAAGTTGT AAATAAATAT AAATAAATAT 600
AACAATCAAA TAACGCACTT ACCTCCTTGA CCACAGCCAA TCCAACGCAA 650
CCGAAAACAG GAGACGGGCC CCGCAAACGC AAATTAAAAT TGCCAACATT 700
ACCGATTATA ACTACAAAAA ACTAACAAGC T                      731
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Fig. 2. Sequence of BglII-HindIII subfragment of Dm665 (0,7 kb) possessing ARS activity. The 11-membered core is framed. Stretches of more than three A's in the enhancer region are underlined. The deletion boundaries are denoted: ◊ - pSL24, Δ - pSL41, ♦ - pSL52.

pSL24, we determined the nucleotide sequence adjacent to the right HindIII site. The deletion boundary in the pSL24 plasmid is at a distance of 379 bp from the HindIII site (Fig.2). In spite of the large size of the deletion, a considerable part of the ARS-containing DNA fragment is preserved. The deletion baundaries in the pSL41 and pSL52 derivatives are located at distances of 106 and 90 bp respectively (Fig.2). Both derivatives have lost the 11 bp core sequence. Nevertheless, these plasmids kept their replication ability in yeast. An inspection of their sequences (Fig.3) shows that the lost core is replaced by AT stretches that resemble it; these stretches have moved, as a result of the deletions from inside the 1.7 kb fragment which does not itself ensure plasmid replication in yeast. Presumably, the sequence retained next to the HindIII site acts as an enhancer of replication, giving rise to a functional ARS when it is approached by the AT stretch, resembling core. If that is true, then the hypotetical enhancer *per se* should not ensure plasmid replication in yeast.

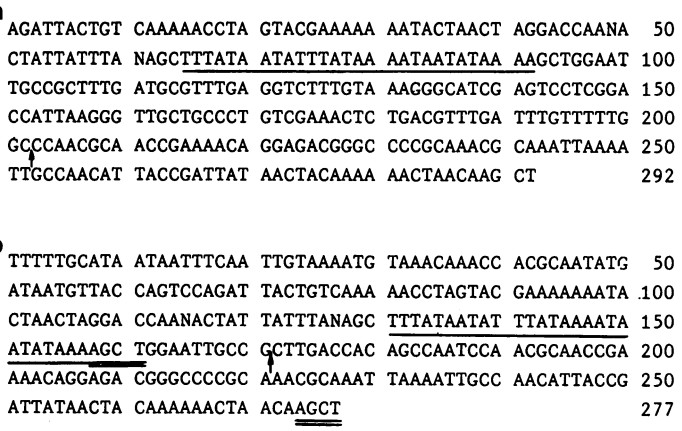

Fig. 3. Sequence of deletion derevatives of the Dm665 fragment. Arrows indicate deletion bounderies. AT-stretches with a structure similar to the core are underlined by a single line, Alu sites - by a double line. "a" - pSL52, "b" - pSL41.

Subcloning of the replication enhancer.

The putative replication enhancer was subcloned from the deletion derivative pSL41 (Fig.3), which has an AluI restriction site at a distance of 119 bp from the HindIII site, making it possible to separate the putative enhancer from the core. The HindIII insert of pSL41 was isolated in a preparative amount and ligated onto itself, thus restoring the AluI site (the AluI endonuclease recognizes four nucleotides within the HindIII site). Then the DNA was restricted with AluI and the mixture of blunt-ended fragments was cloned using BamHI linker in the yeast integrative plasmid YIp5. A plasmid with an insertion of about 120 bp was selected. The sequencing of the insert sonfirmed the cloning of the desired fragment. As we have expected, the plasmid thus constructed did not transform yeast cells, proving the absence of an active ARS from the enhancer fragment.

Sequences homologous to the replication enhancer.

We compared the sequence of the replication enhancer with 14 ARSs obtained from yeast (12,19-21), Drosophila (10,22), Tetrahymena (23) and human DNA (6). Certain short segments of enhancer sequence turned out to be homologous to segments in different ARSs. Since these homology areas are not extensive

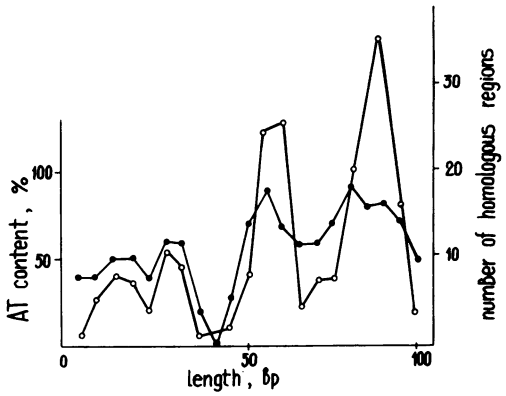

Fig. 4. Comparison of the local AT content of the enhancer sequence and the frequency of occurrence of homologous sites in 14 different ARS elements.
—●— - content of 10 base pairs surrounding a given pair (%)
—○— - number of homology sites with other ARS elements including the given pair.

and are usually confined to AT-rich regions, we tried to assess their significance by comparing the local AT content of the segments of our enhancer sequence with the frequency of occurence of regions homologous to them in other ARSs (Fig.4). A very good correlation suggests that the existance of numerous homologous regions may reflect the generally high AT content of various ARSs.

The anomalous mobility of the ARS-carrying DNA fragment.

Since we have failed to find a sequence that would be common to our enhancer and other ARSs, it is natural to ask what structural features are responsible for enhancer s ability to stimulate replication. Bent DNA regions may be relevant in this connection. They occur whenever there are stretches of more then three A's (or three T's) separated by multiples of 10.5 bp (24). Dm665 sequence (Fig.2) contains such stretches, particularly in the enhancer region. Bent DNA has lower mobility in polyacrilamide gel electrophoresis. The effect is especially pronounced at low temperatures. In 6% rolyacrilamide gel at 5°C the 175-bp fragment containing the subcloned enhancer and the pUC19 polylinker moves as a fragment of 190 bp (Fig.5a). At 30-40°C this mobility shift is clearly smaller (Fig.5b).

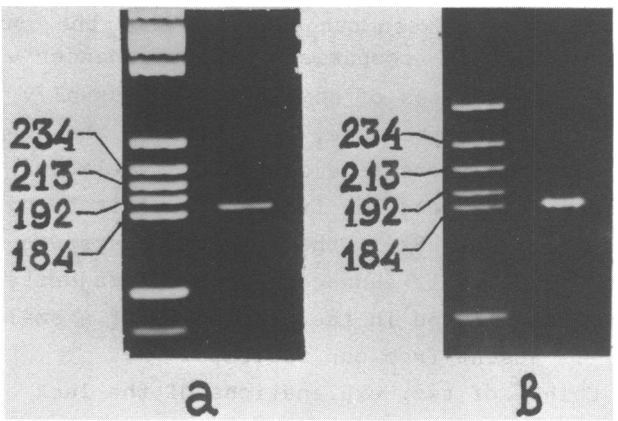

Fig. 5. Mobility of the enhancer-containing fragment in 6% polyacrylamide gel. Electroforesis was performed at 5°C (a) and at 30°C (b). Molecular weght markers - BspRI restricted pBR322.

DISCUSSION

Using subcloning and deletion analysis, we have localized and sequenced the ARS in a previously cloned fragment of Drosophila DNA hybridizing with the telomeres of chromosomes X, 2 and 4. This ARS contains a consensus core sequence and enhancer adjacent at the 5'-end of its T-strand. The data obtained for various ARSs indicate that the core sequence is necessary for replication (3,4). Our results also show that ARS-carrying fragment (0.7 kb) contains the core TAAATATAAAT. The shortest deletion variants do not have it, but it is replaced by an AT-rich regions with a sequence similar to consensus (Fig.3). Since these regions are located within the large HindIII-BglII fragment (1.7 kb) which is not capable of ensuring replication in yeast, they are not suffitient for replication and need to be activated by the adjacent enhancer located next to the right HindIII end of the Dm665 fragment. On the other hand, the enhancer per se does not possess ARS activity.

The subdivision of ARS into core and enhancer has been done by several authors (5-7, 10). The enhancer was always located at the 3'-end of the core s T-rich strand. In our case the enhancer is at the 5'-end i.e. it is possible that the orientation of the enhancer relative to the core is unimportant. We failed to

establish homology between our enhancer and the sequences of various other ARSs. A comparison of our enhancer with any one ARS reveals multiple sites of homology, but usually they belong to AT-rich regions. Furthermore, by virtue of the AT-rich nature of an ARS sequence, there is a distinct correlation between the AT-content of a region and the frequency of it being homologous to sequences in other ARSs. Others in their systems also failed to establish homology of enhancers with the majority of ARSs. The homology region found in the enhancers of a small selection of ARSs (12) is absent from our sequence.

We can think of two explanations of the lack of clear-cut homology between the supposed enhancers of various ARSs. There is a body of data to the effect that plasmids with different ARSs undergo replication in yeast at a certain characteristic times in the S-phase (1). It cannot be ruled out that the different timing of replication is due to the successive appearence of different regulatory proteins each of which has its own binding site at some origins of replication. If this is so, the ARSs may vary significantly as to the structure of enhancers binding these hypotetical proteins. The other possible explanation is that the exact sequence of the enhancer itself is of no matter but it has to ensure certain structural changes essential to replication: a local change of a DNA secondary structure, a bend, a change of chromatin structure, etc. Indeed, in the case of the ARS adjacent to the H4 histon gene of yeast, it has been demonstrated that the core has to be accompanied by 70-bp-long enhancer segment, but the substitution of a linker for any 10 bp of this sequence does not affect the ARS function (9). For ARS1 there is a bend at about 80 bp from the core, and an increase in a distance inactivates the ARS (25). Bent DNA has also been found in the origins of replication of *λ* (26) and SV40 (27). We suppose a similar structure to be present in the sequence of our enhancer.

Does the sequence we have cloned have anything to do with the initiation of DNA replication in Drosophila cells? We do not know the answer, like we do not know it in the case of any ARS cloned from eukaryotic DNA other than yeast. Most likely the answer is no, considering that the sequences ensuring replica-

tion in S.cerevisiae, S.pombe and Chlamydomonas have proved to be different (28,29). One has no grounds for assuming that the specific origins of replication of Drosophila chromosomes (if indeed they exist) are similar to the replication origins of yeast.

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