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Location and Characterization of Autonomously Replicating Sequences from Chromosome VI of *Saccharomyces cerevisiae*

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We have reported the isolation of linking clones of *Hin*dIII and *Eco*RI fragments, altogether spanning a 230-kb continuous stretch of chromosome VI. The presence or absence of autonomously replicating sequence (ARS) activities in all of these fragments has been determined by using ARS searching vectors containing *CEN4*. Nine ARS fragments were identified, and their positions were mapped on the chromosome. Structures essential for and/or stimulative to ARS activity were determined for the ARS fragments by deletions and mutations. The organization of functional elements composed of core and stimulative sequences was found to be variable. Single core sequences were identified in eight of nine ARSs. The remaining ARS (*ARS603*) essential element is composed of two core-like sequences. The lengths of 3′- and 5′-flanking stimulative sequences required for the full activity of ARSs varied from ARS to ARS. Five ARSs required more than 100 bp of the 3′-flanking sequence as stimulative sequences, while not more than 79 bp of the 3′ sequence was required by the other three ARSs. In addition, five ARSs had stimulative sequences varying from 127 to 312 bp in the 5′-flanking region of the core sequence. In general, these stimulative activities were correlated with low local ΔGs of unwinding, suggesting that the low local ΔG of an ARS is an important element for determining the efficiency of initiation of replication of ARS plasmids.

Eukaryotic chromosomes consist of multiple replication units (replicons), which replicate once in the cell cycle in a fixed sequential order (11, 13, 24, 32). The mechanism and regulation of the initiation of replication at the beginning and during the progression of S phase have been the major issue in the study of the molecular biology of eukaryotic DNA replication. By analogy with prokaryotic chromosomes, we would expect eukaryotic replicons to be composed of two regulatory elements involved in the early steps of replication initiation. One is the replication origin, a *cis* element which acts as a signal for initiation of replication, and the second is the initiator protein, a *trans* element which recognizes the *cis* element to initiate the first step of a sequence of reactions leading to the initiation of replication.

Autonomously replicating sequences (ARSs) isolated from *Saccharomyces cerevisiae* are the best candidates so far for the replication origins of chromosomes in eukaryotes. Indeed, some of the yeast ARSs have been shown to behave like origins (4, 27, 42). At the present, two *cis*-acting elements appear to be essential for the function of yeast ARSs. One is an AT-rich 11-bp sequence common to all ARSs, 5′-(A/T)TTTA(C/T)(G/A)TTT(A/T)-3′ (43). So far, all ARSs examined except *ARS121* contain either a perfect match or a 10-of-11-base match to the consensus sequence as elements essential for ARS activity. These sequences are called core sequences, and mutations and small deletions within them completely abolish ARS activity (2, 3, 5, 19, 23, 30, 45) as well as origin activity (7). The other *cis* element is composed of a sequence in the 3′ region of the T-rich strand of the core sequence. These elements are referred to as DNA-unwinding elements (DUEs) and are operationally defined as nuclease-hypersensitive sites (26, 40–42). By analogy with AT-rich 13-mer sequences in *Escherichia coli oriC*, the helical instability of DUEs is thought to be required for initial unwinding of the double helix to permit the entry of replication machinery into the origin of the chromosome (20). The sequential deletion of the DUE has been shown to cause a gradual reduction in replication efficiency and finally the loss of ARS function before the deletion encroaches on the core sequence (26). Computer analysis has shown that the helical instability (local ΔG of unwinding) is correlated well with both ARS efficiency and the nuclease-hypersensitive site (26). DUEs have not been observed in the 5′-flanking region of the core sequence. Other stimulative sequences which can work at a distance from the core sequence have been reported. Of those, the best studied are the OBFI-ABFI binding sites associated with *ARS1* and *ARS121* (8, 23, 38, 45–47).

Most studies of ARSs have been focused on those either related to specific genes (2, 12, 19, 35) or isolated randomly from various *S. cerevisiae* chromosomes (6, 39). The map of the ARS positions on chromosome III has been described recently (15, 28). It is essential to study the complete set of ARSs from a single chromosome systematically in order to understand the role of ARSs in the mechanism and regulation of replication of multireplicon chromosomes. It is now possible to study the replication of an individual chromosome of *S. cerevisiae*, as some of the chromosomes can be physically separated and their primary structure can be analyzed by subsequent cloning and sequencing (29). We have recently reported the construction of a physical map of chromosome VI and the isolation of two sets of linking clones, each of which covers 80% of the chromosome (18).

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The aims of the present work were to isolate a complete set of ARSs from chromosome VI, to map their precise locations on the chromosome, and to characterize their functional structures.

MATERIALS AND METHODS

Strains. *S. cerevisiae* DKD-5D-H (*MAT***a** *leu2 his3 trp1*) was used throughout this study. Transformation of DKD-5D-H was done by the Li-Ac method (17). *E. coli* DH5α [F^- *supE44* Δ *lacU169* (f80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] was used for plasmid preparation. Transformation of *E. coli* was done by electroporation (9).

Plasmids. pKS1 was previously called YIplac128CEN4A (see Fig. 1) (18). In order to invert chromosomal inserts relative to *CEN4* and *oriP* sequences, the multiple cloning site region of pKS1 was replaced by that of pUC18 to create pKS2. pKS3 is the same as pKS1, except that it has the *CEN4* fragment in the direction opposite to that in pKS1. Ylplac128 (14) was used to measure the ARS activity of the *CEN6*-containing fragment.

Construction of a complete clone library and the physical map of chromosome VI were reported previously (17). Sixty-eight *Eco*RI, 75 *Hin*dIII, and 3 *Xba*I fragments and 1 *Eco*RI-*Hin*dIII fragment, which together spanned a continuous 230-kb stretch of chromosome VI, were subcloned into pKS1. To examine the correlation between mitotic stability and doubling time, the following plasmids were constructed. pKS1m*ARS1*A was constructed by inserting the 19-bp core region of *ARS1* (36) at the *Bam*HI site of pKS1. pKS1m *ARS1*B was constructed by inserting the same 19-bp fragment at the *Bam*HI site but oriented in the direction opposite to that in pKS1m*ARS1*A. pKS3m*ARS1*A was constructed by inserting the same 19-bp fragment at the *Bam*HI site of vector pKS3. pKS3m*ARS1*WB was constructed by inserting two copies of the 19-bp sequence at the *Bam*HI site of pKS3. pKS1*ARS1*A was constructed by inserting the 838-bp *Nae*I-*Eco*RV fragment of *ARS1* at the *Sma*I site of pKS1. pKS1 *ARS1*B was constructed by inserting the same fragment in the direction opposite to that in pKS1*ARS1*A.

Generation of deletions and mutations. Deletion derivatives of ARS-containing fragments were prepared by (i) the exonuclease III digestion method, (ii) the preparation of deletion fragments by polymerase chain reaction, or (iii) digestion with appropriate restriction enzymes. The plasmid containing a 30-bp *ARS604* fragment was constructed by inserting the synthetic oligomer

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5'-GATCAATGTTCATTTTACGTTTTGTTACACAAAT-3'
    3'-TTACAAGTAAAATGCAAAACAATGTGTTTACTAG-5'
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into the *Bam*HI site of pKS1 in both directions. Site-directed mutagenesis of all ARSs was performed with the Mutan-G kit obtained from Takara Shuzo Co. following protocols provided by the manufacturer. All mutated plasmids were sequenced to confirm mutation sites or deletion endpoints.

ARS activity and mitotic stability assay. ARS-containing fragments were identified by measuring their ability to transform *S. cerevisiae* DKD-5D-H with efficiencies 10^3-fold higher than that of the control vector plasmid pKS1. In addition to the high-frequency transformation, ARS activity was defined by two criteria: (i) whether transformants could be restreaked on selective plates and (ii) whether transformants could grow in liquid selective medium. The mitotic stability of ARS plasmids was determined by measuring the decrease in the percentage of cells containing a plasmid during incubation for 10 generations in nonselective medium (22). Cells from each colony were inoculated into selective liquid medium and grown until the optical density at 600 nm reached 0.5. The percentage of plasmid-bearing cells was determined by diluting and plating of 100 to 200 cells on a nonselective medium followed by replication on selective plates. This process was repeated after the transformants were diluted into nonselective medium and grown for 10 generations. From the percentage of plasmid-containing cells at generations 0 and 10, mitotic stability (the fraction of cell divisions in which both daughter cells receive plasmids) was calculated (25). For each plasmid, mitotic stability was determined by using two independently isolated transformants.

To compare the ARS activities of various deletion mutants from a given ARS, doubling times of transformants containing the deletion plasmids of the ARS were measured under identical conditions. Cell growth was monitored by reading the optical density of the logarithmically growing cultures at 600 nm at 1-h intervals over a period of 10 h. The data were obtained by using two independently isolated transformants for each plasmid. We used the data with correlation coefficients better than 0.990. For each growth experiment, the percentage of plasmid-containing cells at generation 0 was determined to make sure that the plasmid was maintained extrachromosomally. When the plasmid was integrated into the chromosome, the proportion of plasmid-containing cells at generation 0 became 100%. In contrast, the proportion was <85% when plasmids were present extrachromosomally.

Calculation of local Δ*G* of DNA. The local Δ*G* of unwinding of the DNA, the free energy required for the strand separation of the 100-bp segment of DNA, was calculated by THERMODYN, the computer program developed by Natale et al. (26). All calculations were carried out with a 100-bp size window with 1-bp step movement and parameters corresponding to 10 mM NaCl and 37°C.

Nucleotide sequence accession numbers. The sequences of all ARSs reported in this paper have been registered in GenBank/EMBL with accession numbers D13931 to D13938.

RESULTS

Identification and mapping of ARSs on chromosome VI. We cloned all except a few large *Eco*RI and *Hin*dIII fragments from a 230-kb continuous stretch of chromosome VI into the ARS searching vector pKS1 (Fig. 1). Since pKS1 cannot replicate autonomously in *S. cerevisiae* cells, ARS fragments can be readily identified by measuring the efficiency of transformation by recombinant plasmids containing chromosomal inserts. In some cases, abortive transformants gave rise to very tiny colonies on selective plates at a high frequency. To make certain that ARS plasmids were present in the transformed cells, transformants were tested for growth in the liquid selective medium. Plasmids that gave a high frequency of transformants which were capable of growing in the liquid selective medium were defined as those containing ARSs (see Materials and Methods). The growth rate in the selective medium and the rate of producing segregants (cells without a plasmid) in the nonselective medium varied from transformant to transformant. Since the vector plasmid contains *CEN4*, which confers to plasmids the ability to segregate correctly at each cell cycle, the mitotic stability of the plasmid should directly reflect the efficiency of initiation of plasmid replication. The mitotic stability of a given plasmid should in turn reflect the ARS activity. Furthermore, by measuring doubling times in the selective medium and mitotic stabilities of the transformants

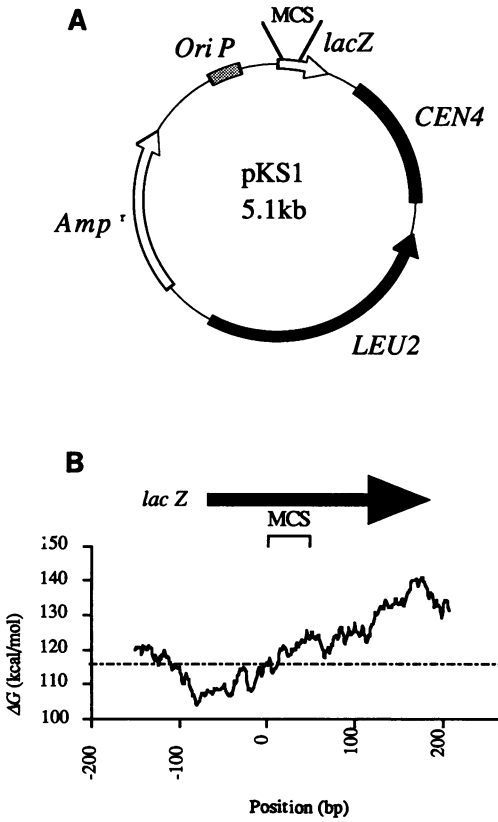

FIG. 1. (A) Structure of vector plasmid used for ARS assay and deletion experiments. The construction of the plasmid is described in Materials and Methods. Other plasmids, pKS2 and pKS3, are derivatives of pKS1 as described in the text. Multiple cloning sites (MCS) (not shown) are (from left to right) *Hin*dIII, *Sph*I, *Pst*I, *Sse*8387I, *Sal*I, *Acc*I, *Hin*cII, *Xba*I, *Bam*HI, *Sma*I, *Kpn*I, *Sac*I, and *Eco*RI. (B) The direction of the *lacZ* open reading frame is shown, with the local ΔG of unwinding near the MCS plotted against the relative position (the *Hin*dIII site is position 0) in the vector. A threshold level of ΔG for the DUE (26) is shown (dotted line). 1 kcal = 4.184 kJ.

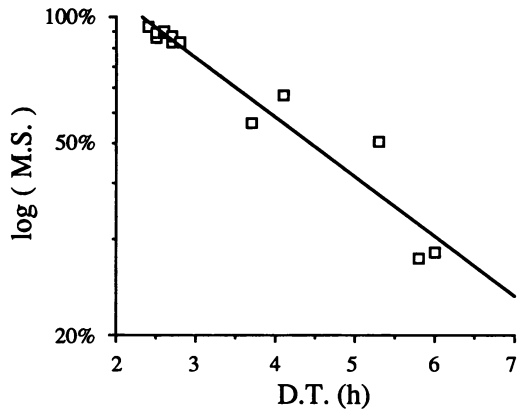

FIG. 2. Correlation of mitotic stability (M.S.) with doubling time (D.T.) of cells bearing an ARS plasmid. The D.T. and M.S. of transformants containing ARS plasmids were determined as described in Materials and Methods. The line of correlation between M.S. and D.T. was constructed by using data for 14 plasmids (see Materials and Methods). The D.T. and M.S. of ARS plasmids for this figure were based on the data in Table 2 and on the following data for various deletion plasmids: plasmid 1D3A (Fig. 4a) had a D.T. of 4.1 h and an M.S. of 67.4%; pKS1m*ARS1*A had a D.T. of 5.8 h and an M.S. of 29%; pKS3m*ARS1*A had a D.T. of 6 h and an M.S. of 30%; pKS3m*ARS1*WB had a D.T. of 5.3 h and an M.S. of 53%; pKS1*ARS1*A had a D.T. of 2.6 h and an M.S. of 92%; and pKS1*ARS1*B had a D.T. of 2.8 h and an M.S. of 88%. The construction of plasmids which contain *ARS1* and its deletion derivatives is described in Materials and Methods.

which carry *ARS-CEN4* plasmids, we found that there was a linear correlation (correlation coefficient of 0.931) between the mitotic stability and the growth rate (Fig. 2). We therefore measured the growth rate of transformants in the selective medium to determine the efficiency of ARSs, particularly when a large number of deletion and linker substitution mutant ARSs were analyzed. When the ARS was fully active, transformants grew with a doubling time of 2.5 h, which is normally observed for wild-type cells in the same medium. Our ARS detection system is very sensitive, because the 19-bp core region of *ARS1* (36) inserted at the *Bam*HI site of pKS1 in either orientation could be detected as an ARS. The doubling times of cells containing the 19-bp insert were 5.8 h for one orientation and 7.8 h for the opposite orientation. Furthermore, a very weak ARS (*ARS604*; see Fig. 4d) was detectable, even when it was deleted to a fragment of 30 bp.

Two restriction fragments, *Eco*RI and *Hin*dIII, were assayed for ARS activity to eliminate the possibility of inactivating an ARS by enzyme digestion. One region, in which both *Eco*RI and *Hin*dIII fragments were too large to be subcloned, was divided by *Xba*I digestion and *Eco*RI-*Hin*dIII double digestion. In total, 68 *Eco*RI, 75 *Hin*dIII, and 3 *Xba*I fragments and 1 *Eco*RI-*Hin*dIII fragment were subcloned and their ARS activities were measured. Table 1 lists all fragments, their sizes and locations on chromosome VI, and the ARS activities of the subcloned fragments. There are four regions in which distances between the nearest *Eco*RI and *Hin*dIII sites are within 100 bp (the minimum distance is 45 bp). ARSs within such regions were detectable by this sensitive assay, even when they were located at the ends of the fragments. In addition, there are 21 restriction sites (6 *Eco*RI, 13 *Hin*dIII, and 2 *Xba*I sites) which have not been covered by other overlapping clones. In these cases, the possibility that some ARSs are located within 30 bp (the size limit of our assay) of the restriction sites remains to be examined.

ARS activities were detected in five *Eco*RI and seven *Hin*dIII fragments and in one *Xba*I fragment. No ARS activities were detected in any other fragments. The positions of these fragments on the physical map of chromosome VI have been determined (18) (Fig. 3). *Eco*RI and *Hin*dIII fragments with ARS activity overlapped in five regions. As for the other two *Hin*dIII ARS fragments, *Eco*RI counterparts were not cloned, because they were too large. A *Hin*dIII ARS fragment at the left end of the cloned region contained two ARSs very close to each other, as described below. Therefore, nine ARSs altogether were identified in the continuous 230-kb stretch of chromosome VI. These ARSs were named, from left to right, *ARS601*, *ARS602*, and so on. The mitotic stabilities and growth rates of the transformants containing these original fragments were determined (Table 2). All cells containing these plasmids except cells with *ARS604* had growth rates equal to that of the wild-type parental cells, indicating that these ARS plasmids are so active that they can replicate at least once per cell

TABLE 1. Fragments cloned for ARS analysis

Fragment no.[a]	Length (kb)	ARS activity[b]	Distance from left end (kb)[c]
E1	0.1	−	10.2
E2	2.4	−	10.3
E3	0.8	−	12.7
E4	0.3	−	13.5
E5	3.2	−	13.8
E6	0.6	−	17.0
E7	7.5	−	17.6
E8	4.1	−	25.1
E9	1.6	−	29.2
E10	1.7	−	30.8
E11	1.2	−	32.5
E12	2.6	−	33.7
E13	0.4	−	36.3
E14	5.0	−	36.7
E15	2.1	+	41.7
E16	2.6	−	43.8
E17	0.3	−	46.4
E18	1.0	−	46.7
E19	1.8	−	47.7
E20	0.4	−	49.5
E21	8.2	−	49.9
E22	7.1	−	58.1
E23	0.5	−	65.2
E24	1.9	−	65.7
E25	11.1	−	67.6
E26	3.2	−	78.7
E28	13.4	Unclonable	81.9
E29	3.0	−	95.3
E30	3.6	+	98.3
E31	0.3	−	101.9
E32	2.8	−	102.2
E33	6.3	Unclonable	105.0
E34	12.0	Unclonable	111.3
E35	0.3	−	123.3
E36	1.0	−	123.6
E37	5.2	−	124.6
E38	0.8	−	129.8
E39	0.2	−	130.6
E40	4.9	−	130.8
E41	0.3	−	135.7
E42	3.3	−	136.0
E43	1.3	+	139.3
E44	6.9	−	140.6
E45	1.0	−	147.5
E46	2.2	−	148.5
E47	0.4	−	150.7
E48	0.1	−	151.1
E49	4.4	−	151.2
E50	1.4	−	155.6
E51	0.2	−	157.0
E52	16.6	Unclonable	157.2
E53	3.8	−	173.8
E54	0.4	−	177.6
E55	2.9	−	178.0
E56	5.0	−	180.9
E57	0.6	−	185.9
E58	0.4	−	186.5
E59	14.8	+	186.9
E60	5.8	−	201.7
E61	2.1	−	207.5
E62	0.6	−	209.6
E63	0.8	−	210.2
E64	0.5	−	211.0
E65	1.1	−	211.5
E66	7.2	−	212.6
E67	0.4	−	219.8
E68	2.4	−	220.2
E69	2.1	−	222.6
E70	1.7	−	224.7
E71	0.3	−	226.4
E72	5.0	+	226.7
H1	3.4	−	0.0
H2	2.7	+	3.4
H3	0.7	−	6.1
H4	7.4	−	6.8
H5	2.9	−	14.2
H6	1.4	−	17.1
H7	1.3	−	18.5
H8	0.3	−	19.8
H9	3.8	Unclonable	20.1
H10	0.2	−	23.9
H11	2.8	−	24.1
H12	3.9	−	26.9
H13	2.2	−	30.8
H14	2.1	−	33.0
H15	1.1	−	35.1
H16	3.4	−	36.2
H17	2.8	+	39.6
H18	1.3	−	42.4
H19	5.6	−	43.7
H20	6.0	−	49.3
H21	0.7	−	55.3
H22	1.7	−	56.0
H23	2.2	Unclonable	57.7
H24	0.8	−	59.9
H25	11.9	−	60.7
H26	0.5	−	72.6
H28	0.1	−	73.1
H29	7.6	−	73.2
H30	4.1	−	80.8
H31	2.9	−	84.9
H32	4.0	−	87.8
H33	2.1	−	91.8
H34	1.0	−	93.9
H35	4.3	−	94.9
H36	1.6	−	99.2
H37	0.2	−	100.8
H38	0.3	−	101.0
H39	0.3	−	101.3
H40	0.3	+	101.6
H41	0.1	−	101.9
H42	2.6	−	102.0
H43	1.5	−	104.6
H44	8.8	Unclonable	106.1
H45	5.8	−	114.9
H46	1.6	−	120.7
H47	0.8	−	122.3
H48	0.3	−	123.1
H49	0.5	−	123.4
H50	0.3	−	123.9
H51	5.3	−	124.2
H52	3.8	−	129.5
H53	5.6	−	133.3
H54	6.2	+	138.9
H55	4.2	−	145.1
H56	0.2	−	149.3
H57	3.4	−	149.5
H58	2.1	−	152.9
H59	3.7	−	155.0
H60	0.6	−	158.7
H61	11.0	−	159.3
H62	1.9	−	170.3
H63	4.2	−	172.2

Continued

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TABLE 1—*Continued*

Fragment no.[a]	Length (kb)	ARS activity[b]	Distance from left end (kb)[c]
H64	1.7	−	176.4
H65	6.2	Unclonable	178.1
H66	2.9	−	184.3
H67	0.4	−	187.2
H68	0.2	+	187.6
H69	0.3	−	187.8
H70	9.7	−	188.1
H71	1.8	−	197.8
H72	4.4	−	199.6
H73	0.2	−	204.0
H74	0.9	−	204.2
H75	1.8	−	205.1
H76	3.0	−	206.9
H77	2.7	Unclonable	209.9
H78	4.5	−	212.6
H79	8.6	−	217.1
H80	2.8	+	225.7
X1	3.8	−	103.7
X2	1.7	−	107.5
X3	4.5	+	109.2
EH1	3.6	−	111.3

[a] Fragments are numbered according to their order on the physical map (18) from left to right. E, *Eco*RI fragment; H, *Hin*dIII fragment; X, *Xba*I fragment; EH, *Eco*RI-*Hin*dIII fragment.
[b] +, ARS activity; −, no ARS activity; Unclonable, fragment was unclonable, so no activity could be detected.
[c] Position of the left end of the fragment.

cycle. Among nine ARSs identified in chromosome VI, only *ARS606* corresponded to one reported previously, *ARS3* (37). These original restriction fragments containing ARSs were used as starting materials for further studies.

Identification of core sequences. To analyze *cis* elements essential and stimulative for ARS activity, it is essential to determine the location of the core sequence in each ARS. The region of the ARS containing the original activity was roughly localized within a 500-bp to 1-kb region in each subcloned fragment by deletional mutation, and the nucleotide sequence was determined. To search candidates for the core sequence of each ARS, the consensus ARS core sequence defined by Van Houten and Newlon, 5′-(A/T)TT TA(C/T)(G/A)TTT(A/T)-3′ (43), was used as a reference. In all nine ARS regions, sequences which matched completely (an 11-of-11-base match) or mismatched by 1 base (a 10-of-11 match) from the core consensus sequence were identified. Mutations were introduced by linker substitution to determine the essential core sequence in each ARS (Table 3). Single core sequences were identified in eight ARSs (Table 3 and Fig. 4). It should be noted that there are two candidate sequences separated by 2 bases in *ARS608*, both of which are 10-of-11 matches and oriented in the same direction (Table 3). Only the right sequence was shown to be a core sequence by mutational analysis (Fig. 4h, 8M1). In the remaining sequence, *ARS603*, both of the candidates, the 10-of-11 and 9-of-11 matches, in the active ARS region were mutated independently without affecting ARS activity (Fig. 4c, 3M1 and 3M2). However, the combined mutation of both of the candidates at the same time completely abolished the ARS activity (Fig. 4c, 3M3), suggesting that these two matches function cooperatively as an essential core element of the ARS.

TABLE 2. Mitotic stabilities of ARSs used for mutation and deletion analyses

ARS	Insert (kb)[a]	Doubling time (h)[b]	Mitotic stability (%)[c]
601, 602	H2 (2.7)	2.6	93
603	H17 (2.8)	2.4	95
604	E30 (3.6)	3.7	58
605	X3 (4.5)	2.7	88
606	E43 (1.3)	2.5	92
607	H62 (1.9)	2.7	91
608	E59 (14.8)	2.5	90
609	H80 (2.8)	2.6	93

[a] Fragments with ARS activity are listed. Fragments are as defined for Table 1.
[b] Doubling time in selective medium.
[c] Mitotic stability of ARS plasmids was determined as described in Materials and Methods.

Effect of vector sequence on ARS activity. To determine the regulatory function of flanking regions of the core sequence on ARS activity, the regions of interest are deleted and replaced by adjoining vector sequences. It is therefore

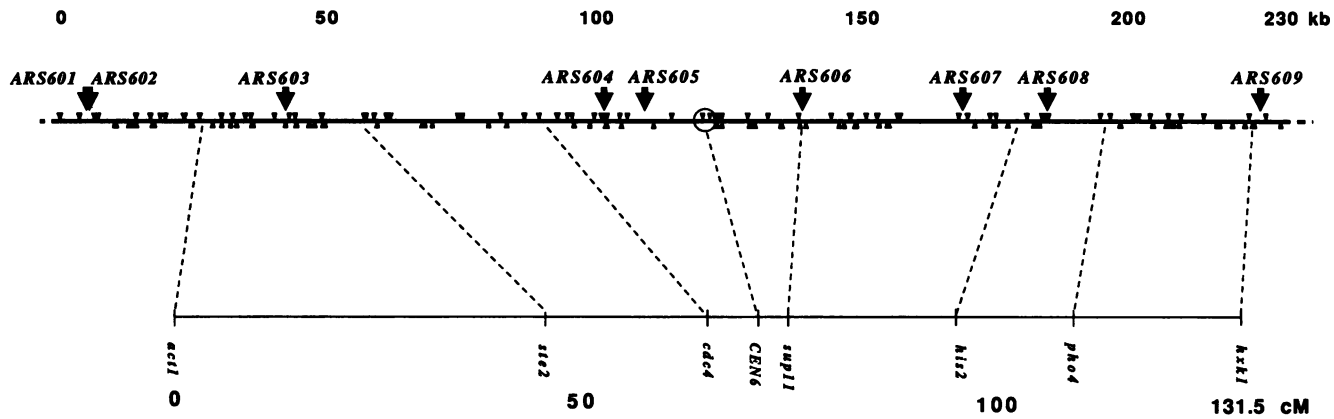

FIG. 3. Contiguous physical map of chromosome VI and locations of ARSs. At the top, the map of *Hin*dIII (triangles above the line) and *Eco*RI (triangles below the line) sites of a 230-kb contiguous stretch of the chromosome VI is shown (18), with locations of ARS fragments listed in Table 2 indicated. At the bottom, the genetic map of the centromere and known genes is shown. cM, centimorgan.

TABLE 3. Oligonucleotides used for creation of mutated plasmids and their effect on ARS activity

ARS	Core sequence candidate	Mutated sequence[a]	ARS activity[b]
601	ATTTCCATTTT	ATcTcgAgTTT	−
602	TTATACGTTTA	TTActCGagTA	−
603	TTTCATATTTT	TcTCgagTTTT	+
	TTTAAAGTTTT	TcTcgAGTTTT	+
604	TTTTACGTTTT	TTTctCGagTT	−
605	AATTACGTTTT	AATctCGagTT	−
606	ATTTATATTTT	ATcTcgAgTTT	−
607	GTTTATATTTA	cTcgAgATTTA	−
608	TTTTACTTTTA	TTTTACcTcgA	−
	ATTTCTATTTT	ATTTCTcgagT	+
609	TTTTATGTTTT	TcTcgaGTTTT	−

[a] Mutagenized bases are indicated by lowercase letters.
[b] +, ARS activity; −, no ARS activity.

important to estimate the contribution by the vector sequence as an environment to ARS activity, particularly when deletions approach the core sequence closely (2). Experimentally, two environments were created by inserting ARS fragments in opposite orientations, A (*oriP* is located close to the 3′ side of the T-rich strand of the core sequence) and B (*CEN4* is located close to the 3′ side of the T-rich strand of the core sequence), to examine the effect of vector sequences (Fig. 1). Estimation of local ΔGs by using the computer program developed by Natale et al. (26) showed that in every case the vector portion had a local ΔG higher than that of the original chromosomal sequence, with the B orientation having the highest ΔG (Fig. 1). As discussed below, the effect of the vector sequence is variable for each core sequence. In general, ARS activities in orientation B are lower than those in orientation A, and this difference becomes larger when deletions are closer to the core sequence. When there were differences in ARS activity between the two orientations, the level of ARS activity decreased most in the original chromosomal fragment, less in the fragment in orientation A, and least in the fragment in orientation B (Fig. 4e and Table 4). However, in some cases (*ARS605*, *-607*, and *-609*; Fig. 4 and Table 4), no differences in ARS activities were observed in any of the three constructs, even when the deletion had nearly reached the core sequence. Judging from the ΔG, the vector sequence in orientation A seems to provide a fairly favorable environment for the core ARS activity and may substitute for part of a chromosomal DUE that has been lost by deletion. In contrast, the sequence in orientation B is rather inhibitory to the core ARS activity. The presence of a stimulative sequence is highly probable when deletion causes a decrease in ARS activities in both orientations. However, the presence of a stimulative sequence is possible but not certain when the decrease in ARS is observed only in orientation B. It is safe to conclude that all stimulative sequences were absent only when a given deletion caused no change of ARS activity in either orientation.

Functional dissection of ARSs. The identification of core sequences by mutation and of flanking stimulative sequences by deletion of each ARS is shown in Fig. 4 and summarized in Table 4. The ΔG of the entire ARS region was calculated (Fig. 4), and correlation with the stimulative sequence was examined.

(i) ***ARS601.*** A single core sequence with a 10-of-11-base match was identified (Fig. 4a, 1M2). The stepwise deletion of the 3′-flanking region revealed a strong stimulative sequence which spanned the region 58 to 277 bp from the core (Fig. 4a, 1D2 to 1D5), and the region showed a low local ΔG. A weak stimulative sequence was also identified (Fig. 4a, 1D3 and 1D7) at the 5′-flanking region corresponding to the region of low local ΔG.

(ii) ***ARS602.*** A single core sequence with a 10-of-11-base match was found to be close to, but on the opposite strand from, *ARS601* (Fig. 4b, 2M2). The stimulative effects of the 3′-flanking region between 55 and 293 bp from the core were not as strong as the effects of the same region on *ARS601* (Fig. 4b, 2D1 to 2D4). A weak stimulative sequence was also identified in a 5′-flanking region which correlated with a low ΔG (Fig. 4b, 2D2 and 2D5). *ARS601* and *-602* are separated by only 241 bp and therefore share parts of the 3′ stimulative sequences. Although the 3′-stimulative sequence of each ARS overlaps the core sequence of the other ARS, the core sequence itself is not directly involved in the stimulation, as the disruption of the core by linker mutation did not affect the stimulative effect on the core of the other ARS (Fig. 4a, 1M1, and Fig. 4b, 2M1). It is interesting that a minimal fragment containing both intact core sequences (Fig. 4a, 1D6A and 1D6B) is less active than fragments containing either of the cores with longer flanking regions (Fig. 4a, 1M1, and Fig. 4b, 2M1).

(iii) ***ARS603.*** No single core sequence was detected. A fragment of 179 bp was as active as the original fragment and contained one 10-of-11-base match and four 9-of-11-base matches (Fig. 4c, 3D5A and 3D5B). Deletion analyses showed that the essential core element was located on a 54-bp fragment which contained only two core-like sequences, one 10-of-11 match and one 9-of-11 match (Fig. 4c, 3D6). Though base substitutions of either of the remaining two matches did not affect ARS activity (Fig. 4c, 3M1 and 3M2), combined mutations of the two abolished the ARS activity completely (Fig. 4c, 3M3), indicating that the two sequences function cooperatively as the essential core element. A 70-bp sequence with an extremely low ΔG located at the 3′-flanking region of the 10-of-11 match was identified as the stimulative sequence for the core element (Fig. 4c, 3D6).

(iv) ***ARS604.*** Although one 11-of-11 match is located on the fragment, the ARS activity was considerably lower than all others, even when the fragment contained an intact 3′-flanking sequence of 429 bp (Fig. 4d, 4D1A and 4D1B). Deletion of the sequence between 192 and 429 bp from the core sequence caused a slight increase in ARS activity, suggesting the presence of an inhibitory sequence in this region (Fig. 4d, 4D1 and 4D2). The additional deletion suggested the presence of strong stimulative sequences in either the 3′- or the 5′-flanking region or in both (Fig. 4d, 4D2 and 4D3). These sequences correspond to the region of low local ΔG.

(v) ***ARS605.*** A single core sequence with a 10-of-11-base match was detected in a fully active fragment of 101 bp (with only 52 bp in the 3′-flanking region) (Fig. 4e, 5D4A and 5D4B), indicating that all *cis* elements required for replication are active, since the original ARS is localized within this region. The deletion experiment revealed a strong stimulative sequence in the 3′-flanking region within 52 bp of the core (Fig. 4e, 5D5A and 5D5B). The decrease in activity caused by the deletion may be explained by the increase in the ΔG of the remaining ARS sequence due to the effect of the flanking vector sequence (Fig. 4e). However, the high ARS activity of the 101-bp fragment cannot be explained by ΔG alone, since the entire region does not have a lower ΔG than the neighboring regions and other ARSs. Deletion of the

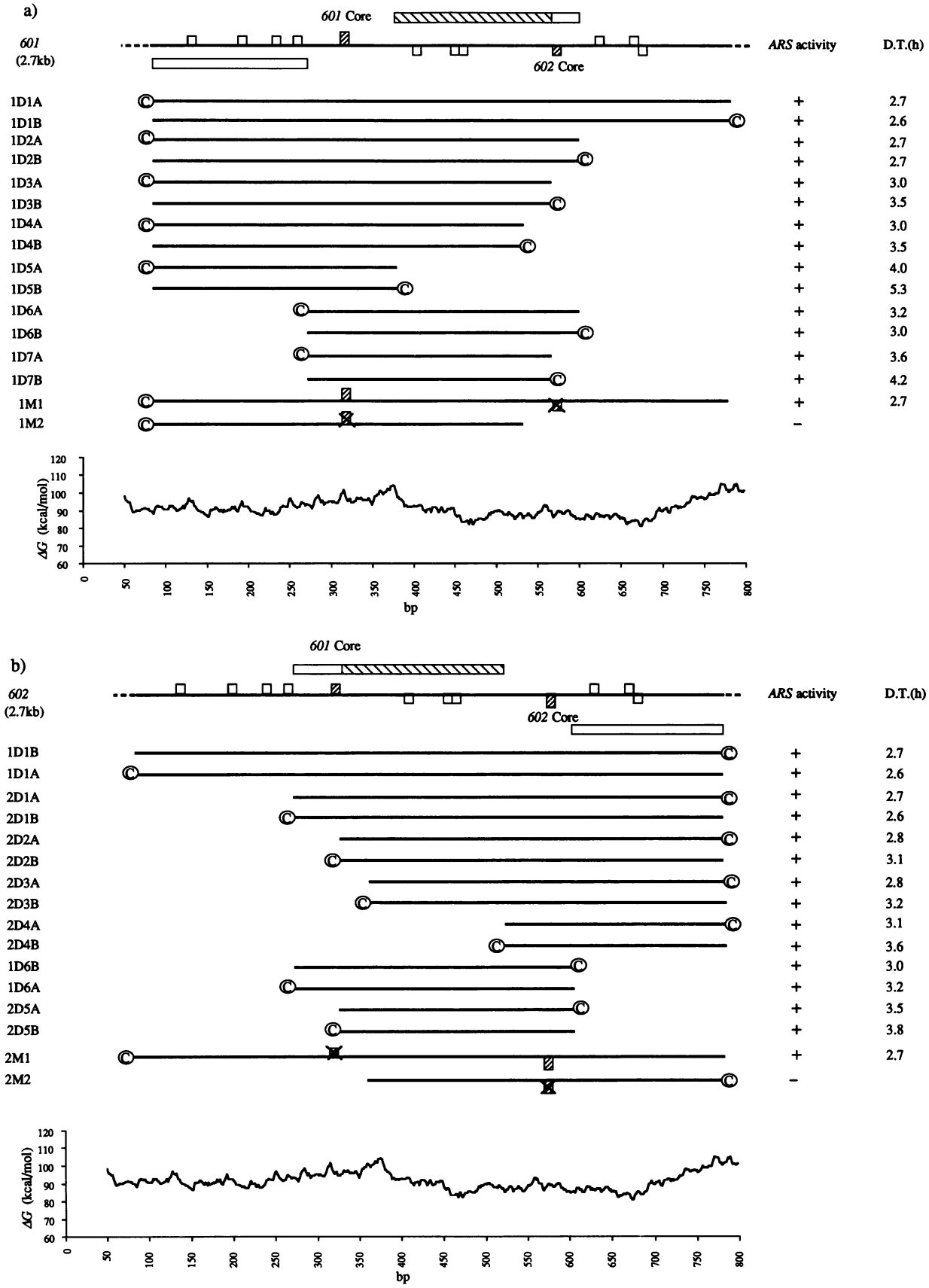
a)
601 Core
601
(2.7kb)
602 Core
ARS activity
D.T.(h)
1D1A + 2.7
1D1B + 2.6
1D2A + 2.7
1D2B + 2.7
1D3A + 3.0
1D3B + 3.5
1D4A + 3.0
1D4B + 3.5
1D5A + 4.0
1D5B + 5.3
1D6A + 3.2
1D6B + 3.0
1D7A + 3.6
1D7B + 4.2
1M1 + 2.7
1M2 -
ΔG (kcal/mol)
bp
b)
601 Core
602
(2.7kb)
602 Core
ARS activity
D.T.(h)
1D1B + 2.7
1D1A + 2.6
2D1A + 2.7
2D1B + 2.6
2D2A + 2.8
2D2B + 3.1
2D3A + 2.8
2D3B + 3.2
2D4A + 3.1
2D4B + 3.6
1D6B + 3.0
1D6A + 3.2
2D5A + 3.5
2D5B + 3.8
2M1 + 2.7
2M2 -
ΔG (kcal/mol)
bp

FIG. 4.

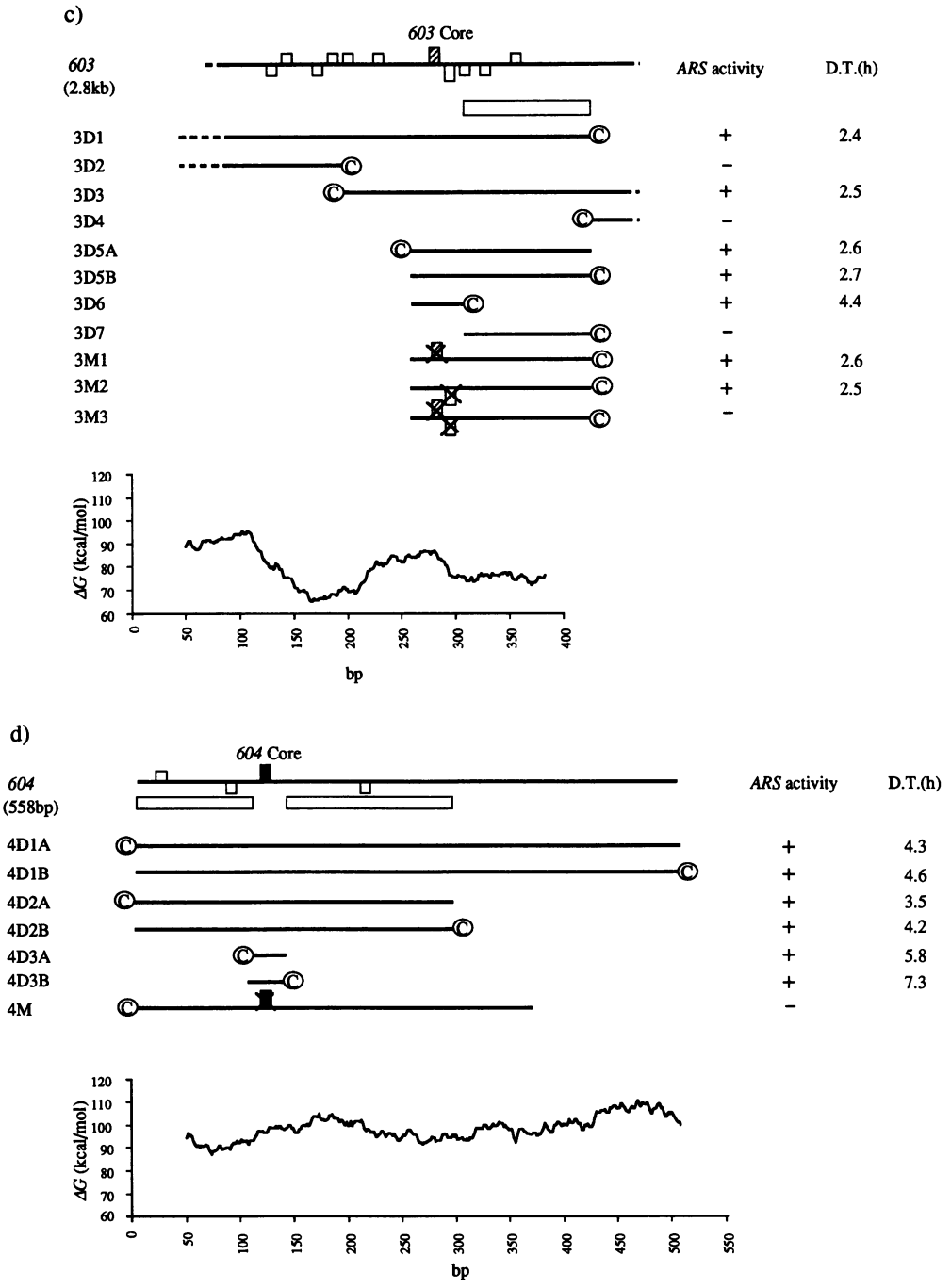

FIG. 4—*Continued.*

5′-flanking region up to 38 bp from the core had no effect on ARS activity.

(vi) ***ARS606.*** A single core sequence with an 11-of-11 match was identified (Fig. 4f, 6M). The stepwise deletion of the 3′ sequence revealed a gradual reduction of ARS activity, suggesting a continuous stretch of stimulative sequence in this region (Fig. 4f, 6D2 to 6D4). A stimulative sequence was also identified in the 5′-flanking region (Fig. 4f, 6D2 and 6D8). Both regions are characterized by low local ΔG.

(vii) ***ARS607.*** A single core sequence with a 10-of-11 match was detected (Fig. 4g, 7M). Deletion of the 3′-flanking region up to 79 bp from the core did not affect the original full ARS activity (Fig. 4g, 7D4A and 7D4B). A slight reduction of the ARS activity was observed within the 79-bp region (Fig. 4f, 7D4 to 7D6) only in orientation B of the insert (Fig. 4g, 7D6A and 7D6B), suggesting the presence of a weak stimulative sequence. The deletion of the 5′-flanking region with a low ΔG up to 21 bp from the core sequence had no effect on the ARS activity (Fig. 4g, 7D7A and 7D7B). This ARS is the second in which the *cis* element required for full ARS activity was shown to be located within a small segment of 111 bp.

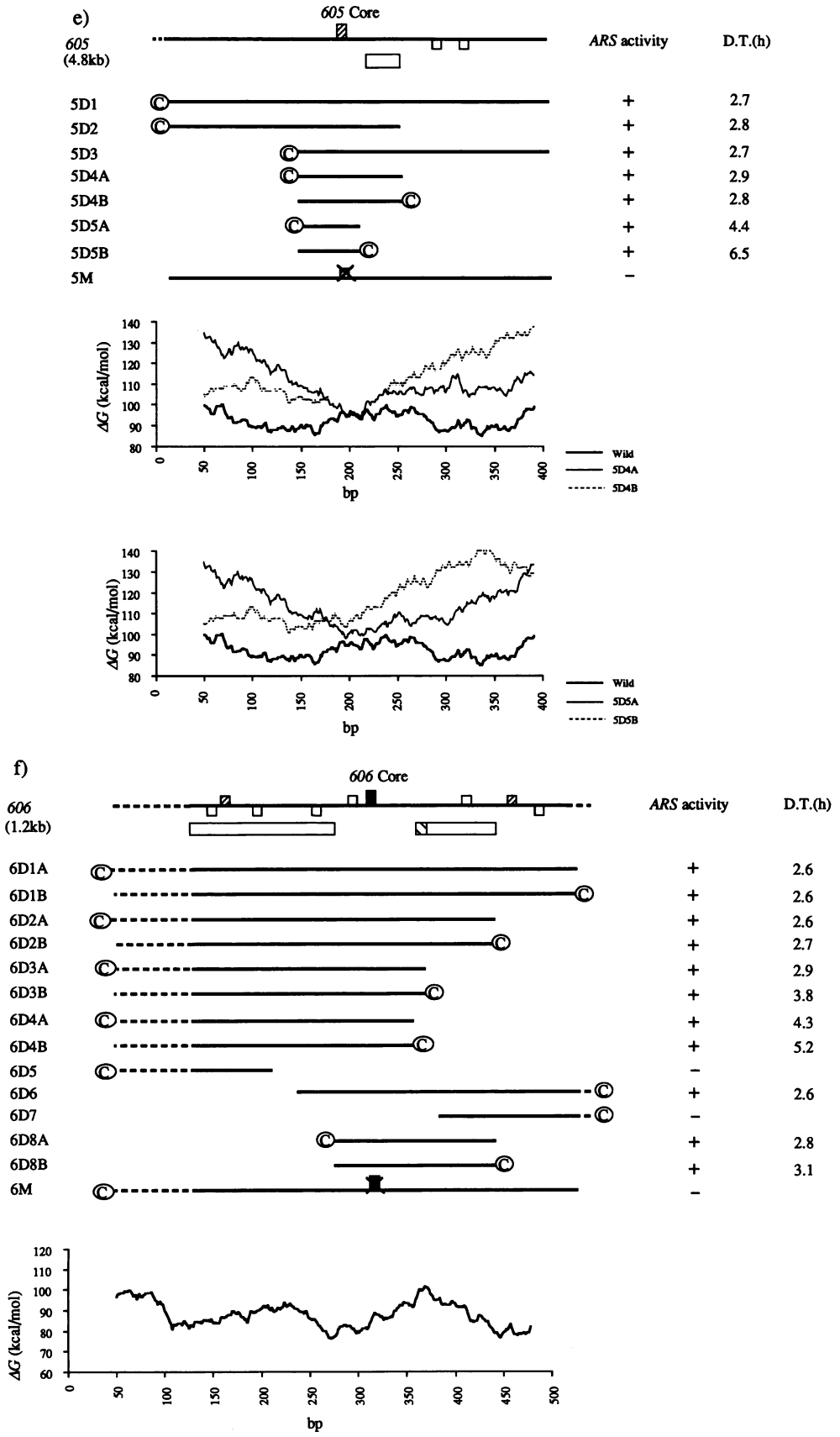

FIG. 4—*Continued.*

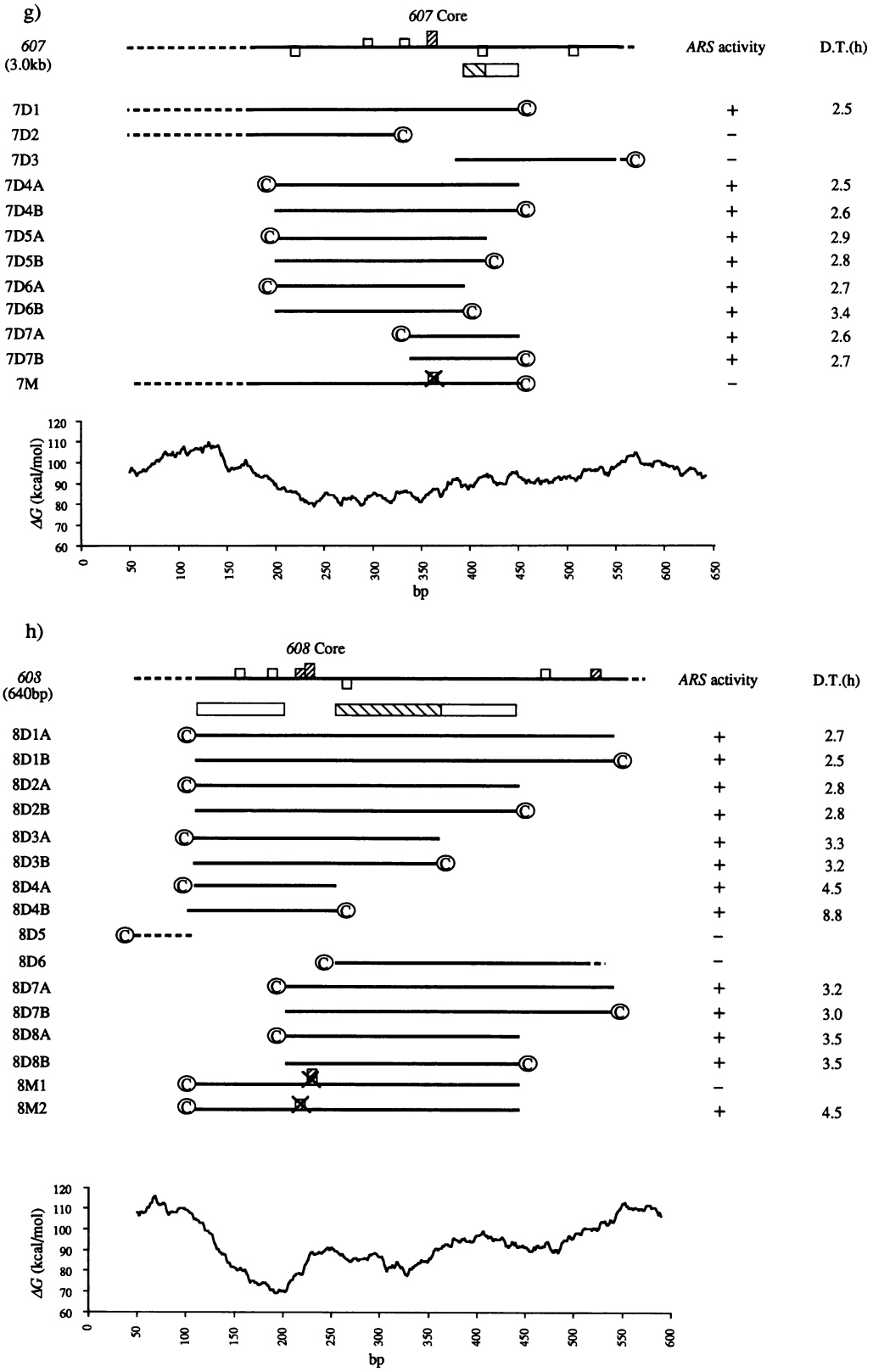

FIG. 4—*Continued.*

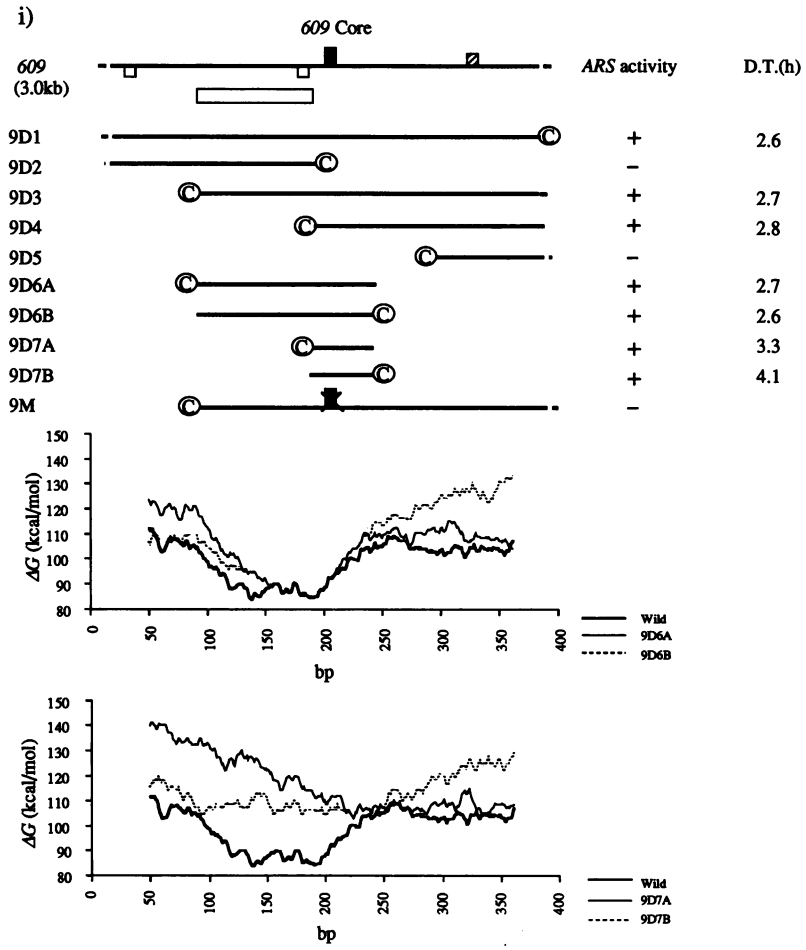

FIG. 4. *cis* elements revealed by deletion and mutational analyses and the local ΔGs of unwinding of nine ARSs. For each ARS (panels a to i), the physical map of core-like sequences, 11-of-11 (filled boxes), 10-of-11 (hatched boxes), and 9-of-11 (open boxes) matches, are shown. The position of the box above or below the line indicates the orientation of the T-rich strand of the core-like sequence, either from 5′ (left) to 3′ (right) or from 3′ (left) to 5′ (right), respectively. The stimulative regions deduced from the results of deletion experiments are shown above or below the map, with the location of the distal end of the stimulative sequence (within the open bar) and the region where deletions caused continuous decreases in ARS activity (shaded bars) indicated. Below the maps are the sizes of deleted fragments and their directions of insertion relative to *CEN4* (circled C). The centromeres are at the left (5′ side) of the fragment in the A orientation and at the right (3′ side) in the B orientation. The amount of starting material used for this analysis is shown below the number of each ARS. Sites of mutation by linker substitution (Table 3) (crosses) are indicated. The definition of the ARS activity of the plasmids and doubling time (D.T.) of the plasmid-containing cells in the selective medium are described in Materials and Methods. Local ΔGs are shown on the graphs below the maps. In *ARS605* and *-609*, ΔGs of *ARS* fragments inserted in the vector plasmids in two orientations, A (solid thin line) and B (dotted line), are overlapped with the original chromosomal segment (solid thick line). 1 kcal = 4.184 kJ.

(viii) ***ARS608.*** A single core sequence with a 10-of-11-base match was identified (Fig. 4h, 8M1). Deletion analysis clearly revealed stimulative sequences in both the 3′-flanking regions (Fig. 4h, 8D2 to 8D4) and the 5′-flanking regions (Fig. 4h, 8D8). Both of these sequences span wide ranges with remarkably low ΔGs of unwinding (Fig. 4h).

(ix) ***ARS609.*** A single core sequence with an 11-of-11 match was detected (Fig. 4i, 9M), and the deletion analysis revealed a stimulative sequence in only the 5′-flanking region (Fig. 4i, 9D7). Deletions of 3′-flanking sequence up to 46 bp from the core sequence showed no effect. This is the third case in which a *cis* element required for full ARS activity was shown to be located within a small segment of 155 bp (Fig. 4i, 9D6A and 9D6B). The segment is characterized by a locally low ΔG, particularly in the 5′ stimulative region (Fig. 4i). Replacement of the stimulative region by vector sequences resulted in the loss of the region with the low local ΔG (Fig. 4i, 9D7).

DISCUSSION

We have identified nine ARSs in the 230-kb continuous stretch of chromosome VI of *S. cerevisiae*. The method used to detect ARS activity was so sensitive that it was possible to detect the 30-bp fragment of the weakest ARS (*ARS604*). Therefore, it is unlikely that some ARSs remain to be detected in the 230-kb stretch of the chromosome, except for the 21 restriction sites which have not been covered by other overlapping fragments. Although the possibility that any ARS is located within 30 bp of those restriction sites is slight, these regions will be tested to complete the search for ARSs on the chromosome. In addition, there remain two telomere-

TABLE 4. Structure and function of deletion derivatives of ARSs of chromosome VI[a]

ARS	Size of fragment showing the indicated ARS activity (doubling time [h])[b]			Core location of stimulative sequence (bases)[c]	
	Full (minimum)	Reduced	Lowest	3′ region	5′ region
601	−235:C:277 (2.7, 2.7)	−235:C:240 (3.0, 3.5) −41:C:277 (3.2, 3.0)	−235:C:58 (4.0, 5.3)	58 to 277	(−41 to −235)
602	−277:C:293 (2.7, 2.6)	−277:C:231 (2.8, 3.1) −25:C:293 (3.0, 3.2)	−25:C:231 (3.5, 3.8)	55 to 293	(−25 to −277)
603[d]	−16:C:152 (2.6, 2.7)	−16:C:27 (4.4)	−16:C:27 (4.4)	(27 to 152)	Not detected outside −16
604	−118:C:192 (3.5, 4.2)	−8:C:11 (5.8, 7.3)	−8:C:11 (5.8, 7.3)	(11 to 192) or (−8 to −118)	
605	−38:C:52 (2.9, 2.8)	−38:C:13 (4.4, 6.5)	−38:C:13 (4.4, 6.5)	(13 to 52)	Not detected outside −38
606	−312:C:112 (2.6, 2.7)	−312:C:55 (2.9, 3.8) −36:C:112 (2.8, 3.1)	−312:C:40 (4.3, 5.2)	40 to 112	(−36 to −312)
607	−21:C:79 (2.6, 2.7)	−178:C:34 (2.7, 3.4)	−178:C:34 (2.7, 3.4)	34 to 79	Not detected outside −21
608	−127:C:210 (2.8, 2.8)	−127:C:126 (3.3, 3.2) −32:C:210 (3.5, 3.5)	−230:C:22 (4.5, 8.8)	22 to 210	(−32 to −127)
609	−98:C:46 (2.7, 2.6)	−13:C:46 (3.3, 4.1)	−13:C:46 (3.3, 4.1)	Not detected outside 46	(−13 to −98)

[a] Data are based on the results shown in Fig. 4.
[b] The size of each deletion fragment is shown by the number of bases on the 5′ (−) and 3′ (+) sides of the 11-bp core sequence (C). Doubling times (given for fragments in orientations A and B, respectively) are for cells containing each deletion ARS plasmid.
[c] Locations in parentheses indicate that distal ends have not yet been determined.
[d] As *ARS603* has no single identifiable core sequence, the location and length of sequence are presented assuming that the 10-of-11 matched sequence to the consensus is the core.

containing terminal regions. In fact, comparison of our restriction map with the map created by Yoshikawa and Isono (48) shows that the distance to the left end is 32.5 kb and that to the right end is 17.5 kb. Judging from the distribution of the nine ARSs in the chromosome, it is unlikely that ARSs other than those associated with the telomeres remain to be detected in the terminal regions. The distribution of the nine ARSs is uneven (Fig. 3). The greatest distance between two ARSs (*ARS603* and *-604*) is 60 kb, and the shortest is 241 bp (between *ARS601* and *-602*). However, in three cases, two ARSs are located fairly close to each other in pairs. If we assume that only one of the two sequences in these pairs functions as the origin of replication at a time, the distribution of origins becomes fairly even, with an average distance of some 45 kb, which is close to the replicon size observed by autoradiograph (31). Recently, 13 ARSs in a 210-kb circular derivative of *S. cerevisiae* chromosome III have been mapped (28). An apparent discrepancy in the density of the ARSs of the two chromosomes is because of the unusually high concentration of ARSs within two specific short segments containing *HML* and *MAT* loci in chromosome III.

To date, detailed analyses of functional elements in the ARS have been performed with only a few ARSs (*H4 ARS*, *ARS307*, *ARS121*, and *ARS1*), from which a general picture of the structure and function of the ARS has been derived (4, 27, 42). However, the systematic analysis of the set of ARSs from a single chromosome, chromosome VI, revealed that basic features are conserved in general but that the organization of functional elements is more variable. Thus, the general feature that the ARS is composed of a core element essential for ARS activity and a stimulative sequence located in the 3′-flanking region of the T-rich strand is observed in most of the nine ARSs. However, there is an exception for the core element, and wide variations in size and location of stimulative sequences were observed.

Regarding the core element, single core sequences vulnerable to base substitutions were identified in eight of nine ARSs. These 11-bp sequences were either an 11-of-11 or a 10-of-11 match to the consensus core sequence proposed by Van Houten and Newlon (43), suggesting that they function as sequence-specific recognition sites for *trans*-acting factors (1) for initiation of replication. As for the sequence specificity, five of nine ARSs on chromosome VI have a C at position 6, while all previously characterized ARSs and the other three sequences examined in this study have a T at this position (Table 3). The significance of the sequence specificity should be examined by site-directed mutation as to the functions of ARSs as chromosomal origins. One exceptional core element was found in the remaining *ARS603*, in which two core-like sequences were required simultaneously as a minimal essential element for ARS activity. The two sequences, a 10-of-11 match and a 9-of-11 match, are located on opposite strands and separated by 5 bp. Since they function cooperatively as the sequence-specific binding sites for *trans*-acting proteins, mutations in either of the sequences may interfere with the binding of the protein to the other site.

Various sequences have been proposed as stimulative *cis* elements for ARS activity. However, no substantial evidence for the elements has been reported, except for the binding sequences for transcriptional protein factors (8, 23, 38, 45–47) and DUEs (26, 40, 41). Deletion analyses have identified stimulative sequences in all nine ARSs on chromosome VI, varying in strength, size, and location. Judging from their nucleotide sequences, these stimulative sequences have no common structure with known protein-binding sites (44). Therefore, the possibility that they are functioning as DUEs was examined by estimating the local ΔG of unwinding with the computer program provided by Natale et al. (26) (Fig. 4). In general, the 3′ stimulative sequences identified in our analyses correspond well to the regions whose ΔGs are lower than those of the neighboring regions. Furthermore, the effect of deletion becomes increasingly serious as the deletion endpoints approach the core sequence. These results suggest strongly that the stimulative sequences are indeed functioning as DUEs.

The size of DUEs is variable. In three cases, *ARS605*, *-607*, and *-609*, fragments as small as 101, 111, and 155 bp, respectively, were isolated as fully active ARSs. In fact, no 3′-flanking DUE was detected within 46 bp of the core sequence of *ARS609*. It is possible to identify the distal endpoints of the stimulative sequence by deletion analysis. However, the presence or absence of DUEs in close prox-

imity to the core sequence is hard to examine by the same technique. In most sequences, including those containing long DUEs, DUEs may be located contiguous with the core sequence and function as essential elements in combination with the sequence-specific functions of the core sequences. The locations of the distal ends of DUEs are variable among ARSs which require long DUEs. It is interesting to examine how these DUEs of various sizes function differentially in a chromosome. In five cases, stimulative sequences were identified in the 5′-flanking regions characterized by low local ΔGs, suggesting that 5′-flanking sequences can also act as DUEs. It should be noted that there is the exceptional case of *ARS605*, in which a short strong stimulative sequence was identified in the 3′-flanking region. The strong stimulative effect cannot be explained by a DUE alone, because the region shows a local ΔG that is higher than those for the neighboring regions. Recently, a *cis* element in the 5′-flanking region was reported to function as a nucleosome positioning sequence (34), which facilitates the opening of the chromatin structure of the nearby core essential sequence.

Eight ARSs (*ARS604* is the exception) are fully active as *cis* elements required for the initiation of replication of plasmid DNA. Regarding the low level of efficiency of *ARS604*, it is interesting that the deletion of part of the 3′-flanking sequence increased the ARS activity. This result suggests the presence of inhibitory sequences which may function as negative regulatory elements for chromosomal replication. It is reported that the efficiency of initiation of chromosomal ARS varies from a constitutively high to a completely silent state (10, 15, 16, 21, 49). Variations in strength and location of stimulative sequences that we observed in nine ARSs may reflect the differences in regulation of the activities of the various chromosomal origins.

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