

Transcriptional regulation of the *Drosophila CycA* gene by the DNA replication-related element (DRE) and DRE binding factor (DREF)

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ABSTRACT

The *Drosophila* gene for cyclin A is expressed in dividing cells throughout development. This expression pattern is similar to those of genes related to DNA replication, suggesting involvement of some common control mechanism(s). In the upstream region (–71 to –64 with respect to the transcription initiation site) of the *CycA* gene, we found a sequence identical to the DNA replication-related element (DRE; 5'-TATCGATA), which is important for high level expression of replication-related genes such as those encoding DNA polymerase α and proliferating cell nuclear antigen. Transient expression assays with chloramphenicol acetyltransferase (CAT) were carried out to examine the function of the DRE sequence of the *CycA* gene. Deletion or base substitution mutations resulted in an extensive reduction in CAT expression. Furthermore, monoclonal antibodies against DRE binding factor (DREF) diminished or supershifted the complex of the DREF and DRE-containing fragment. The results indicate that the *Drosophila CycA* gene is under the control of a DRE/DREF system, as are DNA replication-related genes.

INTRODUCTION

It is now well established in eukaryotes that a number of CDK/cyclin complexes play major roles in cell cycle progression. Cyclin A is first expressed at the G₁–S transition and is required for entry into the S and M phases. Therefore, it may be involved in the regulation of DNA replication (1,2) and also transcriptional control during S phase (3,4). Cyclin A is found in dividing cells throughout development of *Drosophila melanogaster* (5) and its constitutive expression has been associated with tumorigenesis (6,7), while, inversely, abolition of its expression was found to cause growth arrest of cells (8). Thus, the expression profile of the gene encoding cyclin A is similar to those of other proliferation-related genes, such as genes involved in DNA replication.

Eukaryotic genes encoding proteins involved in DNA replication appear to be coordinately expressed in response to signals for cell growth and/or cell cycle progression. Furthermore, common transcription regulatory mechanisms have been found to function in expression of various DNA replication-related genes. For example, replication-related genes of budding yeast are expressed depending on cell cycle progression and a common sequence (*Mlu*I cell cycle box) present in promoter regions of these genes and the specific binding factor DSC1, the complex consisting of products encoded by the *SWI6* and *MBP1* genes, are known to be required for their transient expression at the G₁–S boundary (9,10).

In mammalian cells, the transcription factor E2F binds to the E2F recognition site (5'-TTTCGCGC) and regulates transcription of a group of genes whose products are necessary for cell proliferation (11,12). This includes the genes encoding DNA polymerase α , dihydroforate reductase, thymidine kinase, c-Myc, c-Myb, Cdc2, proliferating cell nuclear antigen (PCNA) and also cyclin A (13–17).

We have isolated *Drosophila* genes for the 180 kDa catalytic polypeptide (18) and 73 kDa subunit polypeptide (19) of DNA polymerase α as well as PCNA (20). The promoters of these genes contain regions featuring a common 8 bp palindromic sequence (5'-TATCGATA), named the DNA replication-related element (DRE) (21). The DRE requirements for promoter activation have been confirmed in both cultured cells (21) and transgenic flies (22). Furthermore, we found a specific DRE binding factor (DREF) consisting of an 80 kDa polypeptide homodimer (21), whose cDNA has recently been cloned (23).

Involvement of DRE/DREF in regulation of a considerable variety of genes has been suggested by the results of DNA database searches (24). It is, therefore, of interest to determine whether the DRE/DREF system is also utilized in the transcription of cell proliferation-related genes other than those directly relevant to DNA replication. To answer this question, we decided to study genes with a role in the cell cycle, because these, like their DNA replication-related counterparts, are expressed dependent on proliferation status. Since the mammalian genes for cyclin A and DNA replication enzymes are commonly controlled by E2F, as mentioned above, we have focused on this gene. A cDNA and

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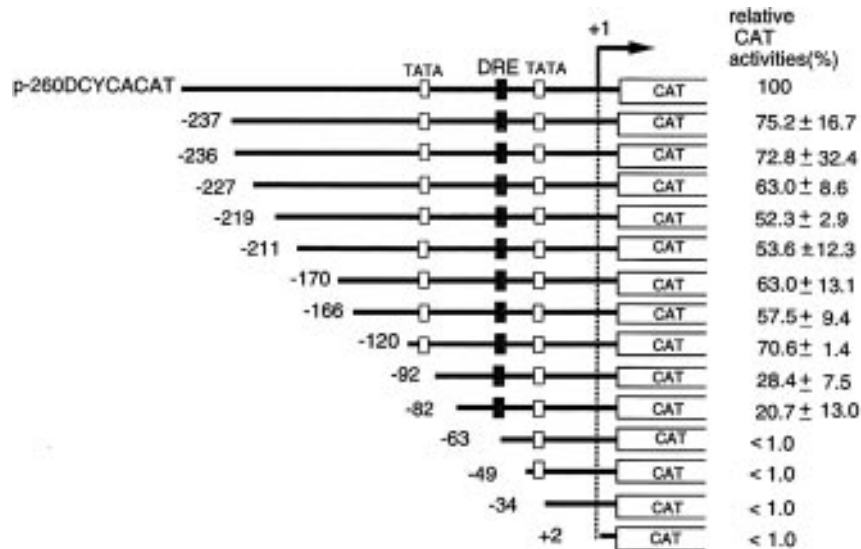


Figure 1. Determination of the regulatory region required for expression of the *Drosophila* *CycA* gene. Features of the reporter CAT plasmid DNA carrying the upstream sequence of the *CycA* gene are schematically at the top. The vertical line with the horizontal arrow indicates the transcription initiation site. The shaded and open boxes indicate the DRE sequence and TATA-like motifs respectively. Two micrograms each of 5'-end deletion derivatives of CAT plasmid DNA were co-transfected with 100 ng luciferase plasmid into Kc cells. Forty eight hours after the transfection, cell extracts were prepared to determine CAT expression levels and values were normalized against the luciferase activities. Averaged values \pm SD obtained from four independent dishes are given as CAT activity relative to that of the wild-type plasmid (p-260DCYCACAT).

the gene for *Drosophila* cyclin A have been cloned and sequenced (5,25). We found a sequence identical to DRE in the region of nucleotide positions -71 to -64 with respect to the transcription initiation site and have examined its role in promoter activity. The obtained results indicate that the *Drosophila* *CycA* gene is indeed under the control of the DRE/DREF system, like DNA replication-related genes.

MATERIALS AND METHODS

Cell culture

Kc cells derived from *D.melanogaster* embryos were grown at 25°C in M(3)BF medium supplemented with 2% fetal calf serum in the presence of 5% CO₂ (26).

Oligonucleotides

To obtain a fragment containing the promoter of the *CycA* gene (nucleotide positions -260 to +12 with respect to the transcription initiation site) by the polymerase chain reaction (PCR), the following primers were chemically synthesized: 5'-ACACTCGAGAAGCT-TAGAATAAATAATATGCAC-3' (containing the region between -260 and -234 and the *Xho*I site); 5'-TTCCGCGGTA-AAAGCAATTGCTGGCTCTTTTTGA-3' (containing the region between -14 and +12 and the *Sac*II site).

The sequences of double-stranded 30 bp oligonucleotides containing the DRE sequence or its base-substituted derivatives in the *CycA* gene promoter were defined as follows:

DRE-CA, 5'-gatccACGACCTATCGATAGCTGGAA-3'

3'-gTGCTGGATAGCTATCGACCTtctag-5';

DRE-CAmut 5'-gatccACGACCTATTCATAGCTGGAA-3'

3'-gTGCTGGATAAGTATCGACCTtctag-5',

where mutated bases are underlined and lower case letters indicate the linker sequence.

The double-stranded 30 bp oligonucleotide DRE-P contains the 24 bp DRE-containing sequence of the PCNA gene promoter and the 6 bp linker sequence, while DRE-PM contains a 2 bp substitution in the DRE sequence of DRE-P (21).

Plasmid construction

To construct the plasmid used for the CAT transient expression assay, a DNA fragment containing the upstream region from position -260 to position +12 of the *CycA* gene was obtained by PCR using *Drosophila* Canton S genomic DNA as a template and the above-defined primer set, digested with *Sal*I and *Sac*II and then placed between the *Sal*I and *Sac*II sites of plasmid pSKCAT (27). The resultant plasmid was named p-260DCYCACAT. A set of 5'-end deletion derivatives of plasmid p-260DCYCACAT were constructed by digestion with *Escherichia coli* exonuclease III and S1 nuclease, as described earlier (28). Deletion break points of these derivatives were determined by nucleotide sequencing.

To construct the plasmids p-260DCYCACATmutI and p-260DCYCACATmutII containing mutations in the DRE sequence, p-260DCYCACAT was digested at the center of the DRE sequence with *Cla*I and then blunt-ended using T4 DNA polymerase, followed by self-ligation using T4 DNA ligase. After this treatment, p-260DCYCACATmutI had an unexpected additional 1 bp at the center of the DRE sequence (TATCCGATA, inserted nucleotide underlined), while p-260DCYCACATmutII had, as expected, an additional 2 bp (TATCCGGATA).

DNA transfection and CAT assay

Kc cells (2×10^6 cells/dish) were grown in 60 mm plastic dishes for 24 h and co-transfected with 2 μ g *CycA* promoter-CAT plasmid as the reporter and 100 ng luciferase plasmid as an

internal control by the calcium phosphate co-precipitation method, as described earlier (29). Cells were harvested 48 h after DNA transfection and cell extracts for determination of CAT activities were prepared as previously reported (30). Radioactivities of spots corresponding to acetylated [¹⁴C]chloramphenicol were quantified with an imaging analyzer BAS2000 (Fuji Film). The luciferase assay was carried out using a PicaGene assay Kit (Toyo Inc Co.) following a documented protocol (31). All assays were performed within the range of concentrations showing a linear relation of activity to incubation time and protein amount. CAT activity was normalized to the luciferase activity.

Gel mobility shift analysis

The gel mobility shift analysis was performed as reported previously (21), with minor modifications. Kc cell nuclear extract and *E. coli* lysate containing GST-DREF(16–608) fusion protein were prepared as described elsewhere (23). These were then added to a reaction mixture containing 15 mM HEPES, pH 7.6, 60 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 12% glycerol, 0.5 µg poly(dI-dC), 0.5 µg sonicated calf thymus DNA (average size 0.2 kb) and double-stranded ³²P-labeled synthetic oligonucleotides (10 000 c.p.m.) and incubated for 15 min on ice. When necessary, unlabeled DNA fragments were added as competitors at this step. DNA-protein complexes were electrophoretically resolved on 4% polyacrylamide gels in 50 mM Tris-borate, pH 8.3, 1 mM EDTA and 2.5% glycerol at 25°C. Gels were dried and autoradiographed.

The gel shift assay was also performed with anti-DREF monoclonal antibody no.1, anti-DREF monoclonal antibody no.4 (23) and anti-chick DNA polymerase α monoclonal antibody 2-4D (32), as a control. Kc cell nuclear extract was mixed with each antibody, incubated for 2 h on ice, added to mixtures containing ³²P-labeled synthetic oligonucleotides (10 000 c.p.m.) and 0.5 µg poly(dI-dC) and then incubated for 15 min on ice as described above.

RESULTS AND DISCUSSION

The presence of a single transcription start site in the *CycA* gene has been determined previously using mRNA from fly bodies at various developmental stages (25). In the upstream region (nucleotide positions -71 to -64 with respect to the transcription initiation site) of the *CycA* gene, we found a sequence identical to the DRE (5'-TATCGATA) (Fig. 1), which is important for the regulation of DNA replication-related genes, such as those encoding DNA polymerase α and PCNA (21).

The genomic sequence corresponding to the -260 to +12 nucleotide positions was amplified by PCR and placed adjacent to and upstream of the CAT gene to construct plasmid p-260DCYCACAT. Transient CAT assays with this plasmid and its 5'-end deletion derivatives were carried out to determine which sequences are important for promoter activity (Fig. 1). An extensive reduction in CAT activity was observed when deletions were extended from position -120 to -92. The deleted region contains a TATA-like sequence (25), although we have not determined that this sequence itself is essential for promoter activation. A further reduction in CAT activity was observed when a deletion extended from position -82 to -63. This region contains the 8 bp palindromic sequence which is identical to DRE (5'-TATCGATA). Since this sequence is important for promoter

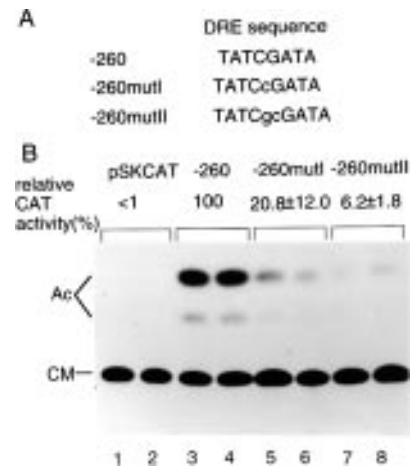


Figure 2. Requirement for the *CycA* DRE for activation of the *CycA* gene promoter. (A) Nucleotide sequences of the *CycA* DRE in the wild-type plasmid (p-260DCYCACAT) and those in the mutant plasmids (p-260DCYCACATmutI and p-260DCYCACATmutII) containing mutations in the DRE sequence are illustrated. Inserted nucleotides are shown by lower case letters. (B) Two micrograms each of CAT plasmids containing the wild-type DRE sequence (*CycA* DRE) or the mutant DREs were co-transfected with 100 ng luciferase plasmid into Kc cells. Forty eight hours after the transfection, cell extracts were prepared to determine CAT expression levels and values were normalized against the luciferase activity. Averaged values ± SD obtained from four independent dishes are given as CAT activity relative to that of the wild-type plasmid (-260, lanes 3 and 4). Acetylated forms of [¹⁴C]chloramphenicol were undetectable in the promoterless CAT (pSKCAT) plasmids included as controls (lanes 1 and 2). Acetylated and non-acetylated forms of [¹⁴C]chloramphenicol are marked by Ac and CM respectively. -260, p-260DCYCACAT; -260mutI, p-260DCYCACATmutI; -260mutII, p-260DCYCACATmutII.

activation and DREF binding, as described later, it was given the name *CycA* DRE.

To investigate the requirement for the *CycA* DRE for activation of the promoter of the *CycA* gene, we introduced 1 and 2 bp insertional mutations at the center of this sequence and carried out CAT assays. The mutations resulted in extensive reductions in CAT activity (Fig. 2), indicating the necessity for an intact sequence for promoter activation.

Gel mobility shift assays were carried out to elucidate the binding of DREF to the *CycA* DRE. When Kc cell nuclear extract was used as the source of DREF, specific DNA-protein complexes could be detected using the chemically synthesized oligonucleotide carrying the *CycA* DRE sequence as a probe (Fig. 3A, lane 1). Complexing with ³²P-labeled DRE-CA was diminished by adding excess amounts of the unlabeled *CycA* DRE-containing oligonucleotide (Fig. 3A, lanes 2 and 3) and DRE-P (Fig. 3A, lanes 6 and 7), an oligonucleotide containing the DRE sequence from the *Drosophila* gene for PCNA (21), as competitors. However, oligonucleotides containing DRE with base-substituted mutations, such as DRE-CAMut (Fig. 3A, lanes 4 and 5) and DRE-PM (Fig. 3A, lanes 8 and 9), did not diminish complex formation. An unrelated sequence of similar size also did not demonstrate any competition (Fig. 3A, lanes 10 and 11). These results indicate that the DRE-CA bound to a specific protein factor, possibly DREF, which is known to bind specifically to DREs from the genes for PCNA and DNA polymerase α (20,21).

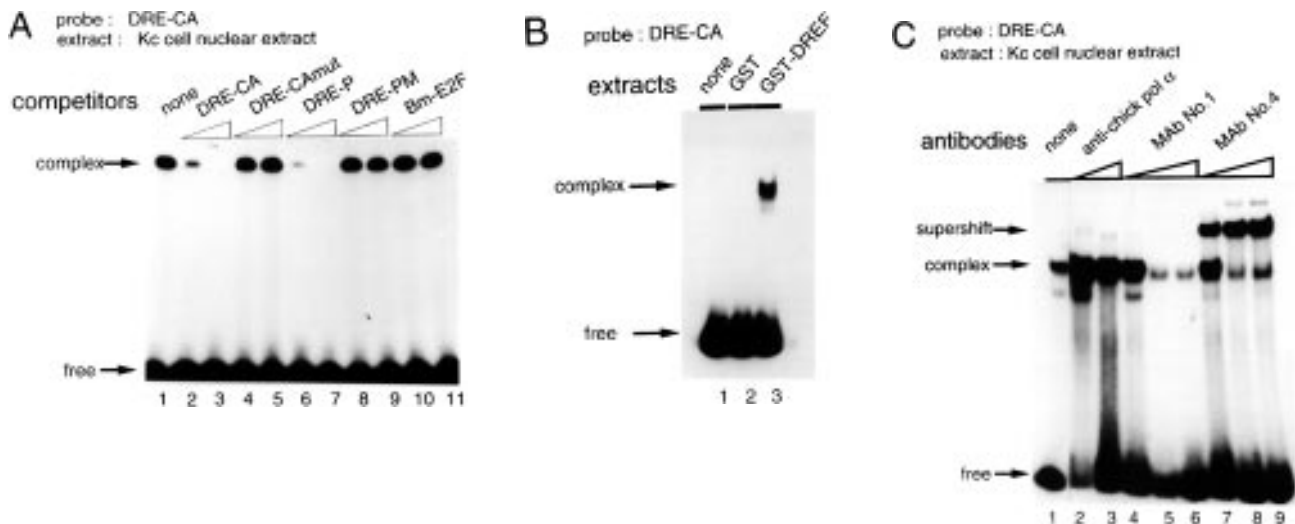


Figure 3. Binding of DREF to the *CycA* DRE sequence. (A) 32 P-labeled double-stranded DRE-CA oligonucleotides were incubated with Kc cell nuclear extract in the presence or absence (none) of competitor oligonucleotides. DRE-CA oligonucleotide containing the *CycA* DRE sequence; DRE-CAmut, oligonucleotide containing a base substituted *CycA* DRE sequence (see Materials and Methods); DRE-P, oligonucleotide containing the DRE sequence from the *Drosophila* PCNA gene; DRE-PM, the DRE-P oligonucleotide having mutations in the DRE sequence; BmE2F, oligonucleotide containing the E2F recognition site from the *Bm.mori* PCNA gene. (B) 32 P-labeled DRE-CA oligonucleotide were incubated with an extract of *E.coli* producing GST-DREF(16-608) fusion protein (GST-DREF, lane 3) or *E.coli* extract containing GST (GST, lane 2). (C) 32 P-labeled DRE-CA oligonucleotides were incubated with Kc cell nuclear extract in the absence (lane 1) or presence (lanes 2-9) of various antibodies: anti-chick pol α , anti-chick DNA polymerase α monoclonal antibody (0.4 and 2 μ l culture supernatant of the hybridoma); MAb No.1, anti-DREF monoclonal antibody no. 1 (0.08, 0.4 and 2 μ l culture supernatant); MAb No. 4, anti-DREF monoclonal antibody no. 4 (0.08, 0.4 and 2 μ l culture supernatant).

DNA-protein complexes were also detected with [32 P]DRE-CA and an extract of *E.coli* producing GST-DREF(16-608) fusion protein (Fig. 3B, lane 3) (23), providing further support for the conclusion that the binding factor is DREF.

In order to confirm the presence of DREF in the complex, we examined the effects of specific antibodies against recombinant DREF (23). As indicated in Figure 3C, the DNA-protein complexes were reduced by monoclonal antibody no. 1 (Fig. 3C, lanes 4-6) and supershifted by monoclonal antibody no. 4 (Fig. 3C, lanes 7-9). The antibody against chick DNA polymerase α applied as a control did not affect the complex formation (Fig. 3C, lanes 2 and 3). Increased amounts of the complex were frequently observed when the culture supernatant of hybridoma cells was added to the reaction (compare lanes 1 and 2). We do not know the reason for this, however, it seems likely that the complex between DRE and DREF is non-specifically protected by protein in culture supernatants. The observed results clearly indicate that a factor containing DREF or DREF itself can bind to the *CycA* DRE.

Our previous studies suggested that the DRE/DREF system plays an important role in transcription of DNA replication genes, such as those encoding the 180 kDa (21) and 73 kDa (19) subunits of DNA polymerase α and PCNA (20). A requirement for this regulatory system was confirmed not only in cultured cells (21), but also in transgenic flies (22). The presently obtained evidence of DRE/DREF involvement in regulation of the *CycA* gene strongly implies that genes other than those directly related to DNA replication may respond to this system. In order to clarify the function of the *CycA* DRE during *Drosophila* development, analyses using transgenic flies carrying the reporter gene under control of the *CycA* gene promoter may be required. Previously, we found that many genes carry DRE sequences within 1 kb upstream of their transcription initiation sites and, therefore, we

hypothesized that they might be under DRE/DREF control (24). The present findings would argue that this hypothesis is correct.

The promoter region of the *CycA* gene contains two TATA-like sequences. Our results indicate the presence of some promoter activating element(s) in the distal TATA-containing region (-120 to -92), although we have not yet established whether the responsible sequence is TATA or some other.

In conclusion, genes encoding proteins with roles in cell cycle regulation may be at least partly in coordination with those responsible for DNA replication, correlating with their common involvement in processes linked to cell proliferation.

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