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Only One of the Origin Binding Forms of SV40 T Antigen has Helicase Activity

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SV40 T antigen exists in monomeric and multimeric forms. We have separated the individual components by glycerol gradient centrifugation. Helicase activity is found to be associated with monomeric forms only. Dimers and other multimeric forms have no discernable helicase activity. However, results obtained from DNA binding experiments carried out with separated forms of T antigen indicate that both monomers and dimers bind to region I and region II of SV40 origin of replication. Possibly monomeric T antigen unwinds DNA at the replication fork while both monomeric and dimeric forms are utilized for positioning of T antigen at the origin of replication.

SV40 is a small DNA virus. For the viral DNA replication in permissive host it requires only one virally coded protein - T antigen (1). The binding of T antigen to the SV40 origin of DNA replication is a prerequisite for the initiation of DNA replication (2-4). T antigen is a 81.5 KD phosphoprotein. Various interesting and important functions have been attributed to the protein (5-13). Among the biochemical properties are its sequence specific DNA binding activity which is stimulated by ATP, intrinsic ATPase activity, nonspecific helicase activity and the related origin dependent unwinding activity. Apparently, T antigen interacts with SV40 origin of replication, and in presence of cellular factors initiate DNA replication utilizing, presumably, the origin specific unwinding activity of the T antigen.

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SV40 T antigen is known to exist in monomeric and different oligomeric forms (14,15). The idea that the process of oligomerization of T antigen may be involved in viral DNA replication has been invoked (16). Several groups have attempted purification of different forms of T antigen by sucrose or glycerol gradient centrifugation (17,18). Both monomers and dimers have been implicated in the sequence specific DNA binding activity. Whereas Gidoni et al. (17) reported the association of DNA binding activity to 5 to 6s form, Bradley et al. (18) found 7s species to have the DNA binding activity and the ATPase activity. Stahl et al. (10) reported that the both the helicase activity and the associated ATPase activity move at 4 to 6s in a 5 to 20% sucrose gradient. However, so far there has been no attempt to correlate SV40 region I and region II binding and helicase activity among the different forms of T antigen.

We have separated different forms of T antigen in a glycerol gradient. Only monomeric form of T antigen has the helicase activity whereas both monomeric and dimeric forms have the ability to bind to origin region I and II of SV40.

Methods

Construction of Plasmids:
The 17 bp SV40 region I sequence GCCTCCAAAAAAGCCTC was synthesized with overlapping cassettes of oligonucleotides in a manner similar to that described by Deb et al.(19). The acceptor molecule is a pML2 derivative (20) and contains sequences 653 through 4361 with a Hind III linker attached to the 653 site and a polylinker Nco I Sal I BamH I Xma I and EcoR I attached to the 4361 end. The phosphorylation of the oligonucleotides, annealing and ligation have been described in detail previously (19). The plasmid pOR-1 has also been described previously (20). It contains SV40 sequences from 5171 through 39 in the same pML2 derived vector background and in between the same restriction sites. SV40 segment has an internal deletion of T antigen binding region I from nucleotide 5178 through 5208 to produce the core origin of replication that overlaps with T antigen binding region II. Transformation of HB101 cells, preparation of cloned plasmid DNA and DNA sequencing have also been described (20).

T Antigen Preparation: CV1 cells were infected with the 776 strain of SV40 (21) at an approximate multiplicity of infection of 10 PFU/cell. After 48 hours T antigen was extracted from the productively infected cells and purified as described by Simanis and Lane (22). The purified protein was dialyzed against 10 mM PIPES [piperazine-N,N'-bis (2-ethanesulfonic acid)], 5 mM NaCl, 0.1 mM EDTA, 1 mM dithioerythritol (DTE), 0.1 mM phenylmethylsulfoniyl fluoride (PMSF) and 10% glycerol, pH 7.0.

Glycerol Gradient Centrifugation: Glycerol gradient centrifugation was done in a manner similar to that described by Bradley et al. (18). Briefly, 30 ug of immunopurified T antigen in 150 ul of 4% glycerol, 20 mM PIPES pH 7.0, 100 mM NaCl, 0.1 mM PMSF and 1 mM DTT were layered on 5 ml gradient of 5 to 40% glycerol in the same buffer. The gradient was centrifuged at 45000 rpm in a Beckman SW 50.1 rotor for 18 hours at 4°C. Approximately 175 ul fractions were collected. Twenty microliters of each fraction was used for each DNA binding and helicase assay and 50 ul was used for SDS polyacrylamide gel electrophoresis. Protein bands in the polyacrylamide gel were visualized by silver staining using an Amersham Kit.

Helicase Assay : Helicase assay was performed following the method described by Stahl et al. (10):

Substrate preparation: Equimolar proportion of M13 mp8 DNA and a 15 mer sequencing primer (3'OH-ATGTTGCAGCACTGA-OH5') were annealed in a buffer containing 10 mM Tris-HCl pH 7.9, 6.6 mM $MgCl_2$ and 60 mM Nacl. The primer was then elongated in the presence of 0.1 mM dCTP, 0.05 mM alpha ^{32}P dATP and 5 mM DTE for 15 minutes at 30°C and another 15 minutes in the same temperature in presence of 1 mM dATP. The labeled substrate was separated from unincorporated nucleotides by gel filtration.

Enzyme assay: The reaction mixture contained 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.5 mM DTE, 2 mM ATP, 0.1 mg/ml BSA and 25 ng of labeled substrate in a total volume of 30 ul. The mixture was incubated at 37°C for 60 minutes. The reaction was stopped with 2 ul of 0.5 M EDTA and the mixture was run on a 12.5% polyacrylamide gel in Tris-borate-EDTA (pH 8.3) buffer for 2 hours at 100 V.

Filter Binding Assay: The binding reactions were done in a total volume of 100 ul containing 20 mM PIPES, pH 7.0, 0.1 mM EDTA, 1 mM DTE, 5-8% glycerol, 0.1 mg/ml BSA, EcoR I-Hinf I labeled DNA fragment and T antigen. Amounts of DNA and T antigen used in individual experiments have been indicated in the Figure. In the binding reactions 150 mM NaCl was used for the fragment from the region I containing clone and 75 mM NaCl was used for the fragment from pOR-1. The reaction mixture was incubated for 1 hour on ice, then filtered through nitrocellulose filters (0.45 u, S & S) and the filter papers were washed with a buffer containing 20 mM PIPES pH 7.0, 0.1 mM EDTA and 75 or 150 mM NaCl. For assaying gradient fractions bound DNAs were eluted from the filters with 10 mM Tris-borate buffer containing 1 mM EDTA, 0.2% SDS, 10% glycerol and 0.05% xylene cyanol and bromphenol blue. Half of the total eluted DNA were ran on a 7.5% polyacrylamide gel.

Results and Discussion

Biological and biochemical studies of SV40 T antigen suggest that this viral gene product is a multifunctional protein which exists in different oligomeric forms. It is not known, however, whether one or all the form(s) perform(s) all the functions or different forms have different biological roles. Specifically, it was not known whether there is any correlation between the form(s) involved in origin binding and the form(s) responsible for helicase function. This is a very important question

since it is reasonable to suspect that there exists a correlation between the T antigen molecules bound to the origin of replication and those responsible for origin specific unwinding activity which again, most probably, depends on nonspecific helicase activity of T antigen. We attempt to answer this question by separating and isolating different forms of the protein on a glycerol gradient and assaying for DNA binding activity and helicase activity in different isolated fractions.

We separated the different forms of T antigen in a linear glycerol gradient to determine whether they differ in their preference for a certain binding region (T antigen binding region I or II) and whether the helicase activity overlaps with one or more particular isolated forms. Immunopurified T antigen was sedimented through a linear 5-40% glycerol gradient. Every individual fraction was tested for region I and region II binding activity, helicase activity and protein content. Figure 1 shows two peaks for region I binding activity, one prominent peak with a small shoulder for region II binding activity and a single peak for helicase activity. There are three protein peaks as pointed out by arrowheads at the top of the Figure. The first peak of region I and region II binding activity, the single prominent peak of helicase activity and the first protein peak are all in the same fraction. The second peak of region I binding activity and the shoulder of region II binding activity are also seen in one fraction, which coincided with the shortest protein peak.

From a plot of 5-40% linear glycerol gradient it was found that the first DNA binding and helicase activity peak, the second peak of region I binding activity and the third protein peak are appearing at 18.5%, 22.5% and 31.8% glycerol concentrations respectively. These glycerol concentrations are reasonably close to the glycerol concentrations where 5s, 7s and 16s forms of T antigen have previously been shown to move (18). These results are also consistent with the observation by Stahl et al. (10) who observed that T antigen associated helicase activity moves 4-6s in a 5-20% linear sucrose gradient. To verify further the s values

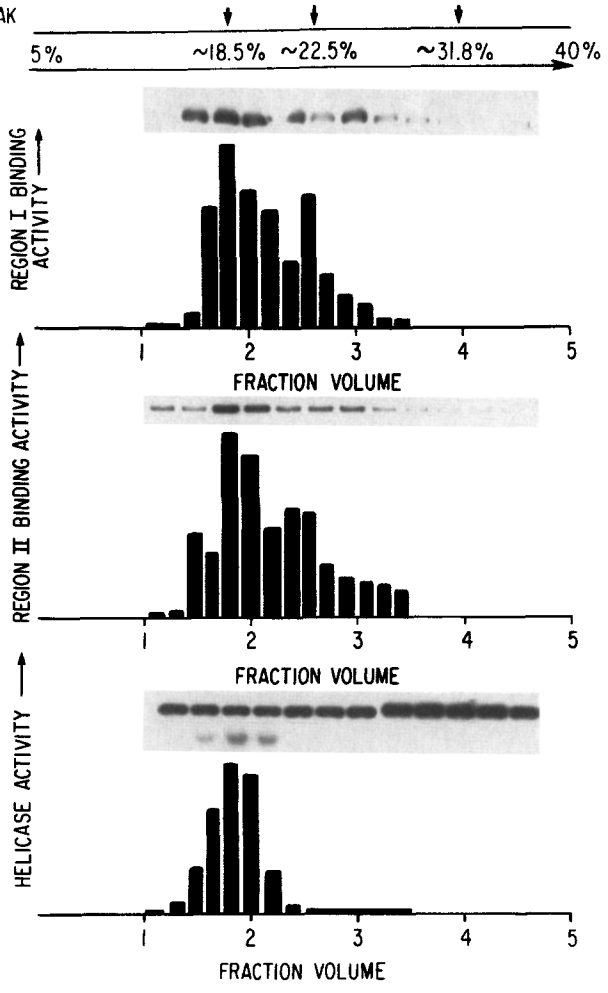

Figure 1

Separation of Different Oligomeric Forms of T Antigen by a Linear 5-40% Glycerol Gradient Centrifugation. Protein content of T antigen was visualized by SDS polyacrylamide gel electrophoresis followed by silver staining. Position of the peaks in the gradient are shown by arrows. Glycerol concentrations of individual fractions were determined from a plot of glycerol concentration vs. fraction volumes and taking into consideration that glycerol concentration at the top was 5% while that at the bottom was 40%. The values of region I binding activity, region II binding activity and helicase activity were obtained by densitometry of the bands on the autoradiogram. Bands shown correspond to fractions 9 to 20 (1.4 ml to 3.5 ml) and densitometry shown from fraction 7 to 20 (1.05 ml to 3.5 ml). Fraction volume is expressed in ml and one fraction was equal to 175ul. The first DNA binding and helicase peak was in fraction 11 and the second DNA binding peak was in fraction 15 - and are not aligned exactly with the fractions in the Figure.

we also ran a parallel glycerol gradient with molecular weight markers phosphorylase b (8.4s) and catalase (11s). From a plot obtained from the movement of the markers in the parallel gradient it again appears that glycerol concentrations of 18.5%, 22.5% and 31.8% correspond to 5s, 7s and 16s respectively. The 5s, 7s and 16s forms of T antigen correspond to

monomers, dimers and tetramers respectively (17,18). These observations indicate that the predominant region I DNA binding form of T antigen is the form moving at 7s (dimers). The 5s form or T antigen has considerable DNA binding activity. Significantly, this monomeric form shows helicase activity. The third form which moved at 16s (tetramer) did not show any appreciable DNA binding or helicase activity.

As mentioned above, fractionation of T antigen on glycerol gradient gave differential helicase and sequence specific DNA binding activity depending on the position of T antigen forms in the gradient. Interestingly, whereas both monomeric and dimeric forms have origin binding activity only monomers have significant helicase activity. What is the biological significance of this differential activities is not yet known. Recent finding that T antigen can unwind SV40 origin containing duplex DNA specifically (11-13) and the finding that T antigen is seen to be present at the replication fork (23-25) suggests interesting possibilities. One of the possibilities could be that positioning of T antigen on the origin of replication can be done by using both monomeric as well as dimeric forms of T antigen. While once positioned only monomeric T antigen is utilized for the origin specific unwinding in the initiation of DNA replication, presumably, utilizing the nonspecific helicase activity of the T antigen monomers. Monomeric forms alone are probably used for helicase activity at the replication fork.

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