An Origin Unwinding Activity Regulates Initiation of DNA Replication During Mammalian Cell Cycle

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An in vitro assay was developed to study the positive factors that regulate the onset of DNA replication during the mammalian cell cycle. Extracts prepared from cells at defined positions in the cell cycle were used to examine the replication of SV40 DNA in a cell free system. Extracts prepared from S phase cells were ten times more efficient at initiating replication at the SV40 origin than were extracts from G_1 cells, whereas elongation rates were similar in G_1 and S reactions. At a discrete point in the cell cycle, just before the cell's entry into S, an activity appeared that was required, in conjunction with SV40 T antigen, for site specific initiation at the SV40 origin. This factor had a role in unwinding DNA at the replication origin.

TO BEGIN TO UNDERSTAND THE events that regulate progression through the cell cycle we have concentrated on one aspect of this process—the cell's "decision" to start DNA replication. Genetic (*1*) and biochemical (*2*) evidence suggest that at some point in G_1 a decision is made, based upon an array of largely unknown specific intra- and extracellular cues, to either commit to DNA replication or withdraw from the cell cycle and enter a quiescent state called G_0.

It is important to distinguish the G_1 to S transition from the mechanisms leading to the cell's return to the cell cycle from the resting G_0 state, the G_0 to S transition. We should not assume that events occurring after the release from G_0 are normal cell cycle events with counterparts in the G_1 to S pathway. The G_0 cell is quiescent, and most cellular activities necessary for growth and division are suppressed; many gene products that are induced after stimulation of growth-arrested cells are constitutively expressed throughout the normal cell cycle (*3, 4*). Cell cycle dependent changes in the abundance of factors necessary for genome duplication have been demonstrated in only a few cases. Transcription of the dihydrofolate reductase (*5*) and histone (*6*) genes increases at the G_1-S boundary, and thymidine kinase activity is similarly cell cycle dependent (*3*). In yeast, many factors involved in DNA replication are induced at start (*7*), but none of the factors analyzed thus far is limiting for the onset of DNA synthesis; the induction of these factors is not necessary for entry into S (*8*). It is not known whether any of the factors in mammalian cells that are induced during DNA replication are rate limiting for the onset of S in the normal cell cycle.

A cell-free system that faithfully replicates DNA molecules containing the SV40 replication origin has been developed (*9*). This system includes (i) exogenous SV40 T antigen as the initiator protein and (ii) a cytoplasmic extract from primate cells that supplies all the other factors necessary for DNA replication such as DNA polymerases α and δ, DNA primase, topoisomerases I and II, DNA ligase, proliferating cell nuclear antigen (PCNA), and a single-stranded DNA binding protein (*10–13*). Since this system relies on cellular factors for all events in DNA replication except those performed by T antigen, we examined cell cycle–associated changes in their activity.

To avoid drug induced artifactual changes in the abundance of replication factors we fractionated cells within the cell cycle by centrifugal elutriation (*14*). This procedure, which is based on the regular increase in cell diameter through the cell cycle, sorts large quantities of cells by the frictional changes associated with increased cell volume. Since this procedure results in fractionation solely on the basis of cell volume, we then determined the cell cycle position of each fraction by flow cytometry, with a fluorescence-activated cell sorter (FACS) 440; this method measured the DNA content of individual cells. Flow cytometry demonstrated that the G_1 and S phase cell populations were contaminated by no more than 2 percent with cells from other positions in the cell cycle. Cytoplasmic extracts were prepared from G_1 and S phase Manca cells (a human Burkitt's lymphoma B cell line), K562 cells (a human

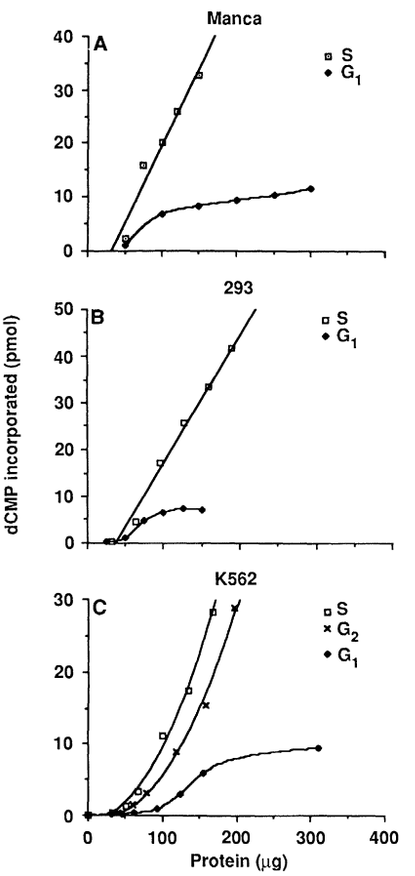

Fig. 1. DNA replication in extracts from G_1, S, and G_2 cells. G_1 and S phase extracts from Manca (**A**), 293 (**B**), or K562 (**C**) cells, and G_2 K562 cell extracts were prepared. Replication reactions were carried out as described (*9*) except that they contained 0.5 μg of T antigen, 100 ng of pSV-ori, and the indicated amount of cytoplasmic extract and were incubated for 1 hour. The T antigen was prepared by immunoaffinity chromatography of cell lysates from Ad5SVR112-infected HeLa cells. Extracts were adjusted to 25 μl with hypotonic buffer plus 100 m*M* NaCl before being added to reaction. Reactions were incubated at 37°C for the times indicated and terminated by the addition of an equal volume of 0.4% SDS, 100 m*M* EDTA. Reactions contained 10 μCi of [α-^{32}P]dCTP (deoxycytidylate), and incorporation of dCMP (decytidine monophosphate) into trichloroacetic acid–insoluble DNA was used to measure the extent of the reactions. The cells (293, K562, and Manca) were grown in spinner culture at densities from 2.5×10^5 to 1×10^6 cells/ml. The 293 cells were grown in Joklik modified suspension medium plus 5% calf serum, and K562 and Manca cells were grown in RPMI medium containing 5% calf serum. Typically, 10^9 cells were separated by centrifugal elutriation (*14*) in a Beckman JE-10X rotor into fractions containing cells in G_1, S, and G_2 phases of the cell cycle. For the cytoplasmic extracts, cells were collected by centrifugation at 1200 rpm (Beckman GPR) for 5 min, washed twice with cold tris-buffered saline (TBS), and resuspended in a hypotonic buffer [20 m*M* Hepes-KOH (*p*H 7.5), 5 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.25 m*M* dithiothreitol] at a density of 10^8 cells/ml. Cells were placed on ice for 10 minutes; Manca and K562 cells lyse spontaneously in this hypotonic buffer and therefore were not washed in this buffer before preparation of cytosolic extracts; the 293 cells were washed twice in hypotonic buffer rather than TBS and were lysed by Dounce homogenization (20 strokes of a type B pestle). After the cells were lysed, the extracts were incubated for 30 minutes on ice. Nuclei and cellular debris were centrifuged for 10 min at 10,000 rpm (Sorvall HB-4); the supernatant (crude cytoplasmic extract) was adjusted to 100 m*M* NaCl and centrifuged at 100,000*g* for 1 hour (Beckman SW55 Ti). The supernatant (S100 cytosolic extract) was frozen at −70°C in 50-μl portions. Protein concentrations were assayed (Biorad assay kit) with bovine serum albumin as a standard and typically were 5 to 12 mg of protein per millimeter of extract.

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erythropoietic cell line), and 293 cells (a human embryonic kidney cell line transformed by the adenovirus E1a and E1b genes).

Our initial experiments demonstrated that cell cycle position determined the ability of cell extracts to replicate SV40 DNA in vitro (Fig. 1). The G_1 and S phase extracts from all three cell types were each used to replicate in vitro a plasmid containing the complete SV40 replication origin, pSV-ori. The amounts of SV40 T antigen, plasmid DNA, and nuceoside triphosphates were adjusted so that the extent of the reaction was dependent only on the amount of cytoplasmic extract. In all cases, replication of closed circular DNA templates was SV40 origin and T antigen dependent, and the results obtained with G_1 and S phase extracts from the three cell types were similar. The specific activity (picomoles of dCMP incorporated per microgram of protein per hour) of S phase extracts was eight to ten times higher than that of G_1 extracts. Since G_1 cells contain about half as much total protein as an S phase cell, the difference in replication activity on a per cell basis was about a factor of 20.

We examined the ability of K562 cells in the G_2 phase of the cell cycle to replicate DNA (Fig. 1C) and found that G_2 cells, which have completed duplication of their genome, continue to express all the factors necessary for DNA replication in vitro. Nuclear transplantation experiments have shown that the G_2 cytoplasm can support ongoing replication in an S phase nucleus (*15*). Our experiments suggest, in addition, that the ability to initiate DNA replication persists as well.

We determined the change in replication activity of cell extracts prepared as cells progressed synchronously from mitosis to S phase (Fig. 2A). The ability of extracts to replicate SV40 DNA in vitro remained low throughout G_1 and suddenly increased 6 hours after mitosis, just as the cells entered S phase. Therefore replication activity does not gradually accumulate during G_1; rather, we have identified a discrete point in the cell cycle when the cell extracts become competent to replicate DNA.

The activation of DNA replication in S phase reactions could have been due either to an increased potential to initiate DNA synthesis or to an increased rate of chain elongation. Experiments that followed the time course of maturation of replication intermediates into fully replicated daughter molecules showed that, once initiated, elongation rates were identical on molecules replicated by G_1 or S phase extracts. Consistent with this observation, we found that many enzymes involved in chain elongation were present equally in G_1 and S phase extracts. Thus the abundance of DNA polymerases α and δ, topoisomerase I, topoisomerase II, DNA ligase, and PCNA did not limit the extent of DNA replication in G_1 extracts (*16*). Furthermore, mixing of G_1 and S phase extracts demonstrated that no inhibitor of replication was present in G_1 extracts. Rather, S phase extracts were able to complement the deficiency in G_1 activity presumably by supplying a limiting replication factor.

The above results implied that the enhanced activity of S phase extracts was due to an increased ability to initiate DNA replication. In this system, de novo initiation of replication on closed circular double-stranded templates is site specific, occurring only at the SV40 replication origin. In contrast, these extracts can also nonspecifically copy any DNA molecule by initiating at nicks or gaps in the DNA template. We tested the ability of extracts prepared hourly as cells progressed from G_1 into S to replicate two different DNA templates. The first template, pSV-ori (*17*), tested the capacity of the extracts to initiate specifically replication at the SV40 replication origin. The second template, nicked calf thymus DNA, tested the capacity of the extracts to support sequence independent initiation at nicks in the template. While the ability to initiate DNA replication at a specific replication origin suddenly appeared at the G_1-S boundary of the cell cycle (Fig. 2A), the ability of α and δ polymerases to replicate DNA nonspecifically decreased slightly through the cell cycle (Fig. 2B). This decline is due to expression of activity measured as per microgram of protein; on a per cell basis there is no apparent change through the cell cycle. These observations suggested that a critical event in the onset of S phase is a change in the cell's capacity for de novo initiation at

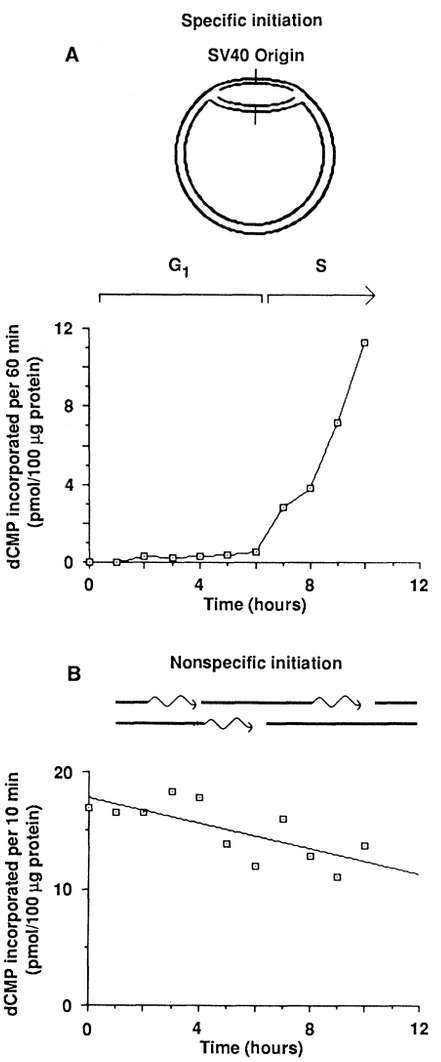

Fig. 2. Cell cycle dependent initiation of DNA synthesis. G_1 cells were prepared by centrifugal elutriation 90 minutes after release of a Manca cell population from nocodazole induced mitotic arrest. Nocodazole treatment was for no more than 10 hours. After elutriation, the residual mitotic cells were discarded, the G_1 cells were returned to culture, and samples were taken hourly for analysis of cellular DNA content by flow cytometry, pulse-labeled with tritiated thymidine to determine when cellular DNA replication (S phase) began, and harvested for preparation of cytoplasmic extracts. Both flow cytometry and thymidine incorporation demonstrate that the Manca cells prepared as described above progress synchronously through G_1 and enter S phase 6 hours after mitosis. In this experiment, crude cytoplasmic extracts, not S-100 extracts, were used in the cell-free replication assay. The extracts were tested for their ability to copy either pSV-ori (**A**) or deoxyribonuclease I treated calf thymus DNA (**B**). For in vivo pulse labeling, 100 μl of labeling mixture containing [^{3}H]thymidine (20 μCi, 84.1 Ci/mmol) in 1 ml of TBS was added to 1 ml of cells (5 to 6 × 10^5 cells per milliliter in complete medium) and incubated for 30 minutes at 37°C. Cells were then diluted with 10 ml of cold TBS and centrifuged at 1200 rpm (Beckman GPR) for 5 minutes, with two additional cycles of washing and sedimenting with 10 ml of cold TBS. The cell pellet was then resuspended in the residual buffer and transferred to a 1.5-ml microfuge tube. The 10-ml tube was rinsed with 1 ml of ice-cold 8% trichloroacetic acid, and the contents were mixed with the resuspended cells. The samples were mixed (Vortex) and incubated on ice for 5 minutes. Precipitates were centrifuged at 15,000*g* for 5 minutes, rinsed twice with ice-cold trichloroacetic acid, and resuspended in 0.2*N* NaOH at 65°C for 5 minutes. Incorporated [^{3}H]thymidine was determined by liquid scintillation counting. For FACS analysis, 10^5 cells were centrifuged (Beckman GPR) at 2000 rpm for 5 minutes. Medium was removed by aspiration, and the cells were resuspended in 100 μl of buffer A (10 m*M* tris-HCl, *p*H 7.4, 30 m*M* NaCl, and 20 m*M* $MgCl_2$); 100 μl of buffer A containing 1% Nonidet-P40 was added, and the suspension was incubated for 5 minutes at 20°C. Then 0.5 ml of propidium iodide (1 mg/ml in TBS) was added, and the samples were incubated for at least 2 hours at room temperature.

the replication origin.

One interpretation of our data was that G_1 cells were deficient in their ability to form sequence specific initiation complexes at the SV40 replication origin. In both prokaryotic cell-free replication systems and in the SV40 system, one of the earliest events in replication of duplex DNA is site specific binding of an initiator protein to the replication origin with subsequent unwinding of the origin DNA by a helicase (*18*). In the SV40 system, both origin binding and the helicase activity are properties of T antigen. However, in addition to T antigen, cellular factors are also required for efficient DNA unwinding at the SV40 replication origin (*19*). To demonstrate directly that initiation was activated in S phase extracts, we have used origin unwinding as an assay for the initiation process.

Increasing amounts of G_1 or S phase extract were tested for their ability to promote T antigen mediated unwinding of the SV40 replication origin (Fig. 3). The amounts of extract used were those that gave a linear increase in the amount of total DNA replication in our standard replication reaction. The difference between G_1 and S phase extracts in their ability to promote origin unwinding was almost precisely the same as the difference in these extracts in their ability to support DNA replication. S phase extracts were ten times more effective at promoting origin unwinding than G_1 extracts. If origin unwinding, as detected here, was a necessary step in the formation of an active replication complex, then this difference was sufficient to explain the increased capacity of S phase extracts to replicate DNA. We have also found that mixing of G_1 with S phase extracts does not inhibit DNA unwinding. Thus, just as for replication, G_1 extracts do not contain an inhibitor of unwinding.

Although it is an early event, an unknown number of steps may precede origin DNA unwinding. Therefore, the extensive origin unwinding stimulated by S phase extracts might be a consequence of activation of a more upstream event, such as more limited unwinding of DNA at the A,T-rich region of the replication origin. In addition we may ask whether the extent of the observed correlation between origin unwinding and DNA replication in vitro reflects a central regulatory switch in vivo. While origin unwinding plays a critical role in replication initiation in other systems (*18, 19*), purification of the S phase activator, by complementation of G_1 replication or unwinding activity, is necessary before we can address these issues definitively. We emphasize that the only difference we have detected between G_1 and S phase extracts is in the ability to unwind DNA at the replication origin. We also point out that S phase extracts are much more active than G_1 extracts even though we add excess deoxy- and ribonucleoside triphosphates to our reactions. Thus, the induction of enzymes involved in nucleotide metabolism (such as thymidine kinase, dihydrofolate reductase, and ribonucleotide reductase) would probably not be sufficient for the onset of S phase in vivo.

Our data suggest that the following sequence of events control the onset of DNA replication during the cell cycle. Most factors necessary to replicate DNA are equally abundant at all phases of the cell cycle. However, the efficient use of these factors to replicate DNA requires that they have functional or physical access to a replication origin. Site specific unwinding of DNA at replication origins, in providing that access, may be the limiting step in DNA replication. One consequence of the cell's decision to begin DNA replication would be synthesis of a factor (or factors) necessary for DNA unwinding at replication origins. This factor is synthesized at the G_1-S boundary.

What is the S phase specific replication activator? Our data indicate that addition of purified human single-stranded DNA binding protein, a factor necessary for efficient origin unwinding (*13, 20, 21*), to G_1 extracts does not stimulate DNA replication and therefore at present there is no evidence to suggest that this protein is a cell cycle dependent replication factor. T antigen not only binds to the origin with high affinity, but in fulfilling its role as a helicase it apparently becomes mobile, abandoning its binding site to move along and unwind the template DNA. In fact, T antigen appears to be a component of the elongating replication fork (*22*), and the T antigen helicase activity is important for continued fork movement

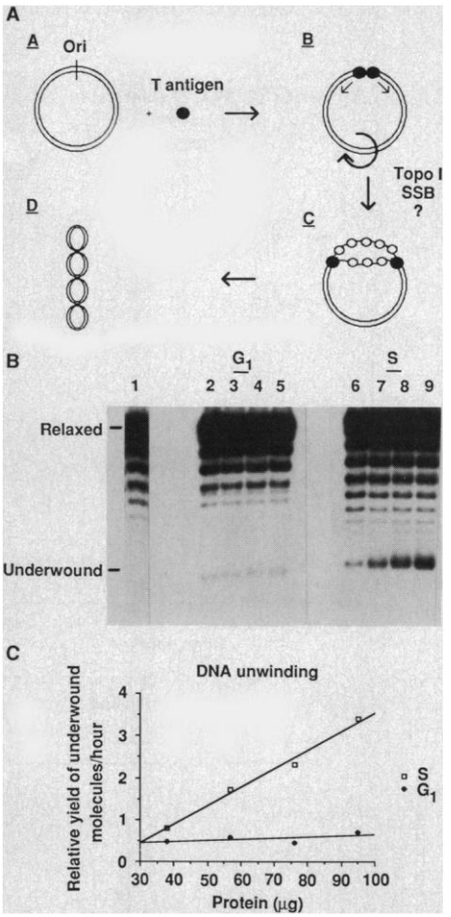

Fig. 3. T antigen dependent origin unwinding. (**A**) A schematic diagram for the pathway of T antigen dependent origin unwinding. T antigen mediated DNA unwinding was assayed by incubating pSV-ori DNA, cell extract, T antigen, ATP, and topoisomerase I at 37°C for 2 hours. During the reaction the template DNA was immediately relaxed by topoisomerase I. Then, as the covalently closed circular DNA was unwound by the T antigen helicase, the compensating positive supercoils induced in the template were removed by the topoisomerase. In this reaction, the cell extract supplied a single-stranded DNA binding protein that stabilized the unwound DNA, thereby promoting extensive unwinding by T antigen. After deproteinization, as the product became fully base-paired over the formerly unwound region, the DNA became negatively supercoiled, with the number of negative supercoils dependent on the number of bases unwound by the T antigen helicase. (**B**) DNA unwinding in G_1 and S phase extracts. The substrate DNA was labeled with ^{32}P for autoradiography. These reactions were performed in 50 m*M* NaCl (that is, replication conditions). (Lane 1) Control reaction containing T antigen, but no cytoplasmic extract; (lanes 2 to 5), T antigen plus 38, 57, 76, or 95 μg of G_1 extract, respectively; (lanes 6 to 9) T antigen plus 38, 57, 76, or 95 μg of S phase extract, respectively. The control reactions demonstrate that the unwinding reaction is T antigen dependent, but that the addition of exogenous topoisomerase is not necessary, consistent with our observation that the S100 extracts contain 0.25 unit of topoisomerase I per microgram of protein. Although the addition of exogenous topoisomerase I is not necessary, we always include it in our reactions to ensure that it is present in excess. (**C**) The region of the gel shown in (B) containing the underwound product was cut out and the relative yield of product was calculated from the ^{32}P label present in each band. Origin unwinding reactions (50 μl) were carried out under standard replication conditions at 50 m*M* NaCl, except that deoxy- and ribonucleoside triphosphates were omitted. The reaction was supplemented with 3 m*M* adenosine triphosphate, 10 units of calf thymus topoisomerase I (BRL), 0.3 μg of T antigen, 2 ng of ^{32}P-labeled pSV-ori DNA and 100 ng of unlabeled pSV-ori DNA (labeled substrate DNA was prepared by purifying the products of a standard replication reaction), and 40 to 100 μg of cytosolic extract. Reactions were incubated for 2 hours at 37°C, stopped by the addition of an equal volume of 0.4% SDS, 100 m*M* EDTA, and digested with proteinase K for 30 minutes at 37°C. Samples were extracted with phenol and chloroform, precipitated with ethanol, and analyzed by electrophoresis through 1% agarose gels in tris-borate-EDTA (TBE). Reaction products were visualized by autoradiography.

(*23*). We suggest that a second cellular contribution to the unwinding reaction is a factor involved in modulating the functional state of T antigen—changing it from a static DNA binding protein to a mobile helicase. This factor may be the S phase activator.

We realize that we have presented the above model without reference to the cellular equivalent of T antigen—the cellular initiator protein. Cyclic control of initiator protein abundance may not be required to regulate effectively the onset of DNA replication. In support of this, we have demonstrated previously, in a specific model system, that in vivo the initiator protein SV40 T antigen remains stably associated with its template DNA for at least two cell cycles (*24*). Despite the continuous association of the initiator protein with its template, the model replicon replicated just once per cell cycle. Thus, the onset of replication was not regulated by the physical presence of the initiator protein (that is, T antigen). Similarly, it is possible that the cellular initiator is constitutively present and stably associated with replication origins. When the decision to replicate DNA is made, a factor that is necessary for the initiator to unwind DNA at the origin is synthesized. Only then does DNA replication begin.

REFERENCES AND NOTES

1. L. Hartwell, R. Mortimer, J. Culotti, M. Culotti, *Genetics* **74**, 267 (1973); L. Hartwell, J. Culotti, J. Pringle, B. Reid, *Science* **183**, 46 (1974); L. Hereford and L. Hartwell, *J. Mol. Biol.* **84**, 445 (1974).
2. A. Pardee, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1286 (1974); A. Yen and A. Pardee, *Exp. Cell Res.* **116**, 103 (1978); M. Mendenhall, C. Jones, S. Reed, *Cell* **50**, 927 (1987); V. Simanis and P. Nurse, *ibid.* **45**, 261 (1986).
3. C. Thompson, P. Challoner, P. Neiman, M. Groudine, *Nature* **314**, 363 (1985); J. Sherley and T. Kelly, *J. Biol. Chem.* **263**, 8350 (1988).
4. R. Baserga, *Exp. Cell Res.* **151**, 1 (1984); *The Biology of Cell Reproduction* (Harvard Univ. Press, Cambridge, MA, 1985); C. Thompson, P. Challoner, P. Neiman, M. Groudine, *Nature* **319**, 374 (1986); D. Prescott, *Intern. Rev. Cytol.* **100**, 93 (1987).
5. P. Farnham and R. Schimke, *J. Biol. Chem.* **260**, 7675 (1985).
6. N. Heintz and R. Roeder, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2713 (1984).
7. L. Johnston, J. White, A. Johnson, G. Lucchini, P. Plevani, *Nucleic Acids Res.* **15**, 5017 (1987).
8. B. Beyers, unpublished observations.
9. J. Li and T. Kelly, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6973 (1984); B. Stillman and Y. Gluzman, *Mol. Cell. Biol.* **5**, 2051 (1985).
10. Y. Murakami *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2869 (1986).
11. G. Prelich, M. Kostura, D. Marshak, M. Mathews, B. Stillman, *Nature* **326**, 517 (1987).
12. L. Yang, M. Wold, J. Li, T. Kelly, L. Liu, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 950 (1987).
13. R. Wobbe *et al.*, *ibid.*, p. 1834.
14. M. Meistrich, in *Cell Separation: Methods and Selected Applications*, T. Pretlow II and T. Pretlow, Eds. (Academic Press, New York, 1983), vol. 2, p. 33.
15. N. DeTerra, *Proc. Natl. Acad. Sci. U.S.A.* **57**, 607 (1967).
16. Activity of α and δ DNA polymerases in G_1 and S phase Manca cell extracts was measured by incubating the indicated amount of extract with activated calf thymus DNA for 10 minutes. Reactions were at least 80% inhibited by Aphidicolin at 10 μg/ml. Most of the cellular DNA polymerase activity was present in our cytoplasmic extracts. DNA polymerase activity was measured under standard replication conditions except that T antigen was omitted, and 5 μg of activated calf thymus DNA was substituted for p-SVori DNA as template. Activated DNA was prepared by incubating 500 μg of calf thymus DNA with 100 ng of deoxyribonuclease I in 10 m*M* tris (*p*H 7.4), 10 m*M* $MgCl_2$ for 10 minutes at 37°C [U. Hubscher, P. Gerschwiler, G. McMaster, *EMBO J.* **1**, 1513 (1982)]. Topoisomerase was measured by relaxation of supercoiled DNA. Reaction products were visualized by ultraviolet illumination. One unit of activity relaxes 0.5 μg of supercoiled DNA in 1 hour at 37°C. Visualization of reaction products on gels in saturating amounts of ethidium bromide indicated that the relaxation of supercoiled DNA in our reactions yielded closed circular, not nicked circular, products demonstrating that we were assaying a nicking-closing activity and not a nuclease. Also, relaxation was not inhibited by the addition of EDTA to the reaction. Both S and G_1 extracts contain approximately 0.25 unit of topoisomerase I per microgram of cell protein (as anticipated, most of the cellular topoisomerase I was not extracted in the preparation of our hypotonic cell lysates). Addition of purified PCNA (*11*) to G_1 reactions did not stimulate replication activity, demonstrating that the abundance of PCNA was not limiting the extent of G_1 replication. Visualization of replication products on agarose gels demonstrated that G_1 reactions did not preferentially accumulate replication intermediates such as catenated dimers or nicked circles, an indication that neither topoisomerase II nor DNA ligase was limiting.
17. J. Roberts and H. Weintraub, *Cell* **46**, 741 (1986).
18. F. Dean *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 16 (1987); M. Dodson, F. Dean, P. Bullock, H. Echols, J. Hurwitz, *Science* **238**, 964 (1987); A. Kornberg, *J. Biol. Chem.* **263**, 1 (1988); H. Stahl, P. Droge, R. Knippers, *EMBO J.* **5**, 1939 (1986); D. Bramhill and A. Kornberg, *Cell* **52**, 743 (1988); T. Baker, S. Sekimizu, B. Funnell, A. Kornberg, *ibid.* **45**, 53 (1986); M. Dodson *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7638 (1986).
19. R. Umek and D. Kowalski, *Cell* **52**, 559 (1988).
20. M. Wold, J. Li, T. Kelly, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3643 (1987).
21. M. Fairman and B. Stillman, *EMBO J.* **7**, 1211 (1988).
22. H. Stahl and R. Knippers, *J. Virol.* **47**, 65 (1983).
23. H. Stahl, P. Droge, H. Zentgraf, R. Knippers, *ibid.* **54**, 473 (1985); L. Tack and G. Proctor, *J. Biol. Chem.* **262**, 6399 (1987).
24. J. Roberts and H. Weintraub, *Cell* **52**, 397 (1988).

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Zinc-Dependent Structure of a Single-Finger Domain of Yeast ADR1

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In the proposed "zinc finger" DNA-binding motif, each repeat unit binds a zinc metal ion through invariant Cys and His residues and this drives the folding of each 30-residue unit into an independent nucleic acid–binding domain. To obtain structural information, we synthesized single and double zinc finger peptides from the yeast transcription activator ADR1, and assessed the metal-binding and DNA-binding properties of these peptides, as well as the solution structure of the metal-stabilized domains, with the use of a variety of spectroscopic techniques. A single zinc finger can exist as an independent structure sufficient for zinc-dependent DNA binding. An experimentally determined model of the single finger is proposed that is consistent with circular dichroism, one- and two-dimensional nuclear magnetic resonance, and visual spectroscopy of the single-finger peptide reconstituted in the presence of zinc.

THE ZINC FINGER MOTIF IS PRESENT in several transcription regulatory proteins (*1, 2*). The model for this protein domain was first proposed based on sequence analysis (*3, 4*), partial proteolysis (*3*), and zinc content (*3*) of *Xenopus* transcription factor TFIIIA. ADR1 is a positive transcription activator of the glucose-repressible alcohol dehydrogenase gene (*ADH2*) in the yeast *Saccharomyces cerevisiae* (*5*). The *ADR1* gene codes for a 1323-residue protein, and sequence analysis has revealed two zinc finger domains between residues 100 and 160 in the protein (*6*). In a genetic analysis of *ADR1* (*7*), point mutations producing null alleles were clustered in the zinc finger sequences and also indicated that specific conserved residues within its two adjacent finger domains were essential for protein function. Recently, an ADR1–β-galactosidase (ADR1–β-gal) fusion protein containing the first 229 residues of ADR1 was shown to footprint both upstream activator sequence 1 (UAS1) and upstream activator sequence 2 (UAS2) of

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