Preferential Binding of DNA Primase to the Nuclear Matrix in HeLa Cells*

(Received for publication, January 28, 1986)

Samuel H. Wood and James M. Collins†

From the Department of Biochemistry, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

Studies of the spatial organization of DNA replication have provided increasing evidence of the importance of the nuclear matrix. We have previously reported a relationship between rates of DNA synthesis and the differential binding of DNA polymerase α to the nuclear matrix during the S-phase. We now report the detection of DNA primase bound to the HeLa nuclear matrix. Matrix-bound primase was measured both indirectly, by the incorporation of [32P]dAMP into the nuclear matrix over the S-phase, and directly, by the incorporation of [3H]dAMP into matrix DNA. Characteristics of this system include a requirement for ATP, inhibition by adenosine 5’-O-(thiotriphosphate), a primase inhibitor, and insensitivity to aphidicolin and α-amanitin, inhibitors of polymerase α and RNA polymerase, respectively. Subcellular quantification of primase and polymerase α activity revealed that while most (~72%) primase activity is located on the nuclear matrix, a majority (~32%) of polymerase α activity is matrix-bound. Treatment of the nuclear matrix with β-D-octylglucoside allowed the solubilization of ~54% of primase activity and ~39% of the polymerase α activity. This data provides further evidence of a structural and functional role for the nuclear matrix in DNA replication. The ability to solubilize matrix-bound replicative enzymes may prove to be an important tool in the elucidation of the spatial organization of DNA replication.

Study of DNA polymerase α, the main mammalian replicative enzyme, has revealed it to be structurally and functionally heterogeneous. Although much of the evidence for heterogeneity is based on differences in chromatographic behavior (e.g., Krauss and Linn, 1982), evidence for functional differences among polymerase α species, based on a variable association with primase, has been adduced. Yagura et al. (1982) isolated two forms of polymerase α, only one of which had associated primase. This form, able to utilize single-stranded DNA templates, was designated “replicase.” A similar polymerase α-primase complex has been immunopurified from human KB cells (Wang et al., 1983, 1984) and calf thymus (Chang et al., 1984). The enzymatic functions in this complex are tightly associated and have resisted efforts at separation.

As these investigations have continued, inquiries into the spatial organization of eukaryotic replication have provided increasing evidence for the importance of the nuclear matrix in this process (Berezney and Bucholtz, 1981; Pardoll et al., 1980; Smith and Berezney, 1982; Valenzuela et al., 1983). This nucleoprotein scaffolding, which remains following high-salt and nuclease treatment of the nucleus (Berezney, 1980), is capable of in vitro DNA synthesis (Smith and Berezney, 1982) and has been demonstrated to be the site at which the majority of nascent DNA synthesis occurs (Pardoll et al., 1980). DNA polymerases, including polymerase α, have been detected on the nuclear matrix (Smith and Berezney, 1983; Richter et al., 1980) and we have recently demonstrated a relationship between the rate of DNA synthesis and levels of matrix-bound polymerase (Foster and Collins, 1985).

In the present report, we provide an initial basis for the integration of these two lines of research by localizing the majority of primase, and thus replicase, to the nuclear matrix. We demonstrate that, while only a minority of polymerase α is located on the nuclear matrix, a majority of primase is so localized. In addition, we report the solubilization of polymerase α and primase by detergent treatment of the nuclear matrix.

**EXPERIMENTAL PROCEDURES**

**Materials**—All tissue culture supplies were obtained from Flow Laboratories. HeLa cells were obtained from Dr. Thoru Pederson, Worcester Foundation for Experimental Biology, Shrewsbury, MA. [2,8-3H]ATP (32 Ci/mmol) and [α-32P]dATP (550 Ci/mmol) were obtained from ICN. [methyl-3H]TTTP (80 Ci/mmol) was obtained from New England Nuclear. ATP, deoxynucleoside triphosphates, dithiothreitol (DTT), β-mercaptoethanol (βME), dideoxyadenosine triphosphate (ddATP), β,α-dithio-dideoxyctylcylate, calf thymus DNA, α-amanitin, and aphidicolin were obtained from Sigma. Triton X-100 was from Eastman. Poly(dT) was from Pharmacia. Escherichia coli DNA polymerase I (Klenow fragment) was obtained from United States Biochemicals. ATP·y·S was from Boehringer Mannheim. Activated calf thymus DNA was prepared as described previously (Foster and Collins, 1985).

**Cell Culture**—HeLa (0.5 × 10⁶ cells/ml) were maintained in spinner culture at 37 °C. Cells were fed every 48 h with Joklik's modified Eagle's minimal essential medium containing 10% fetal calf serum and 1.25 μg/ml of Fungizone (Collins, 1978).

Preparation of Nuclei and Cytoplast—Nuclei and cytoplast were isolated as described previously (Foster and Collins, 1985). HeLa cells (2–4 × 10⁶/ml) were held for 16 h at 4 °C in 25 ml of a buffer containing 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, and 1 mM MgCl₂. The cells were disrupted by a Dounce homogenizer (~30 strokes) such that essentially no intact cells were noted by microscopic visualization. The nuclei were isolated as the pellet, and the cytoplast as the supernatant, following centrifugation of this homogenate at 10,000 rpm in a Sorvall RC2-A, Type SS-34 rotor for 10 min.

Preparation of Nuclear Matrix—Isolation of the nuclear matrix was accomplished by a modification of the method of Pardoll et al., 1980. Nuclei, isolated as described above, following the 16-h period during which endogenous nuclease digestions occur (Foster and Collins, 1986), were resuspended in 10 ml of TMP (10 mM Tris-HCl, pH 7.5, 0.2 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride). Nuclei were salt-extracted by the slow addition over 1 h of 4 ml of a 5 M NaCl/Tris·HCl solution to a final 2 M NaCl concentration. Following

*The abbreviations used are: DTT, dithiothreitol; βME, β-mercaptoethanol; ATP·y·S, adenosine 5’-O-(thiotriphosphate); PBS, phosphate-buffered saline; ddATP, dideoxyadenosine triphosphate.

†To whom requests for reprints should be sent.
centrifugation of this homogenate (as described above for nuclear isolation), the pellet was washed three times with phosphate-buffered saline (PBS). Following the final wash, the matrices were suspended in 10 ml of PBS prior to analysis. For some experiments, including those examining the possibility of nonspecific protein binding to the matrix, washed nuclear matrix was subject to extensive additional washing. Specifically, nuclear matrix preparations were washed an additional four times with a low-salt buffer (20 mM KPO₄, pH 7.5, 2 mM βME), then three times with a high-salt buffer (0.5 M KPO₄, 2 mM βME), and finally three times with the low-salt buffer.

**Solubilization of Matrix-bound Primase and Polymerase α**—Following incubation in the low-salt buffer for 24 h at 0 °C, the extensively washed nuclear matrix preparation was sonicated five times for 15 s at 20-s intervals and allowed to remain on ice for 2 h. This homogenate was then incubated with 0.5 M KCl and 22 mM β-octylglucoside for 1 h at 0 °C. Subsequent to centrifugation at 40,000 rpm in a Beckman L2-65 centrifuge, Type 65 rotor at 4 °C, the supernatant and pellet were collected. The pellet, representing residual matrix, was washed three times with PBS then resuspended in 5 ml of PBS. The supernatant, containing solubilized matrix components, was then extensively dialyzed against PBS.

**Enzyme Assays**—Polymerase activity was measured by a method modified from Edenberg et al. (1978). Incorporation of [³²P]dTMP into activated DNA was measured in a reaction mixture (250 μl) containing 10 mM Tris·HCl, pH 8.0, 10 mM MgAc, 2 mM DTT, 0.67 mg/ml of bovine serum albumin, each of dATP, dGTP, dCTP, [³²P]dTTP (1 μCi/ml), 100 μM dATP, and 50 μM of the sample to be assayed. Incusion of dADP under these conditions provides a greater than 85% inhibition of polymerase β. After incubation at 37 °C for 30 min, the reaction was terminated by the addition of 5 ml of cold 1 N HCl, 1% NaPP₃. The reaction mixture was collected on Whatman GF/C filters, washed successively with ml of cold 1 N HCl, 15% NaPP₃, and 3 ml of cold 90% ethanol, and the radioactivity was determined in a scintillation counter. Under the conditions described, incorporation of labeled TMP is linear over 60 min. One unit of polymerase α activity is defined as that amount which catalyzes the incorporation of 1 nmol of TMP into activated DNA in 1 h at 37 °C. Primase was assayed in a coupled reaction involving the incorporation of labeled dAMP into poly(dT), a single-stranded template, by polymerase α and the Klenow fragment of E. coli polymerase I (hereafter referred to as the Klenow fragment) following the formation of the requisite ribonucleotide primer by primase. The reaction mixture (250 μl) consists of the following components: 25 mM Tris·HCl, pH 8.0, 2 mM MgCl₂, 4 mM DTT, 50 μM [³²P]dATP, 25 μM [α-³²P]dATP (3 Ci/mmol), Klenow fragment (1 unit/ml), 200 μg/ml bovine serum albumin, and 50 μl of the material to be assayed. Modifications of this assay for primase is a coupled one which involves the indirect detection of ribonucleotide primer formation through the measurement of deoxyribonucleotide incorporation on a single-stranded template. Since the coupled enzymes, polymerase α and the Klenow fragment of DNA polymerase I, are unable to utilize unprimed DNA templates, any deoxyribonucleotide incorporation with these templates is assumed to follow primer formation by primase. Detection of matrix primate activity using this assay is complicated by the presence on the matrix of an endogenous DNA synthesis system (Foster and Collins, 1985) and its associated residual matrix DNA which potentially could serve as a template for the Klenow fragment. The properties of the matrix poly(dT) utilizing system were examined by varying the components present in the assay system (Table I). In the absence of nuclear matrix, the Klenow fragment is unable to use poly(dT) as a template. That the low level of “endogenous” activity seen (minus Klenow, minus poly(dT) condition) is due in large part to polymerase α is indicated by the inhibition seen with the addition of aphidicolin, a potent inhibitor. The lack of stimulation by poly(dT) in the presence of aphidicolin would seem to indicate that in the absence of the Klenow fragment, primer extension is due to matrix-bound polymerase α. The absence of poly(dT) from the “complete” system results in ~15% of maximal activity.

**RESULTS**

**Detection of a Poly(dT) Utilizing System on the Nuclear Matrix**—The ability of the nuclear matrix to utilize a poly(dT) template is demonstrated in Fig. 1. In the absence of labeled poly(dT), incorporation of labeled dATP is seen only over the first 5 min. With increasing poly(dT) concentration, progressively more dATP is incorporated until, under the conditions used in subsequent experiments (50 μg/ml), a 6-fold increase in activity is seen.

**Properties of the Matrix Poly(dT) Utilizing System**—As mentioned under “Experimental Procedures,” the standard assay for primase is a coupled one which involves the indirect detection of ribonucleotide primer formation through the measurement of deoxyribonucleotide incorporation on a single-stranded template.
This reflects, in part, the capacity of the Klenow fragment to incorporate deoxyribonucleotides into matrix DNA. In all subsequent experiments, matrix primase activity was defined as that activity in the complete system minus that activity seen in the absence of poly(dT). This provides a conservative estimate of matrix-bound primase activity as it does not include that activity which results from de novo primer formation on matrix DNA. This matrix primase system has several similarities to soluble primase. Little primase activity is seen in the absence of added ATP. Primase activity exhibited a 55% inhibition with the primase inhibitor, ATPγS, a value consistent with that noted by Yagura et al. (1982). Control experiments revealed no inhibition of Klenow fragment or matrix-bound polymerase α activity by ATPγS (data not shown). Primase activity was completely resistant to α-amantidine (0.5 mg/ml), a potent inhibitor of RNA polymerases II and III (Roeder, 1976).

Incorporation of Ribonucleotides into Matrix DNA—It is also possible to directly measure the incorporation of [3H] ATP into matrix DNA. A time course of this reaction is seen in Fig. 2. α-Amanitine (0.5 mg/ml) inhibits this activity by no more than 5% (data not shown), indicating that under these conditions only a small fraction of the putative primase activity would be due to the RNA polymerase activity noted to be present on the matrix by other investigators (e.g. Ciejć et al., 1982; Jackson and Cook, 1985).

Subcellular Localization of Primase Activity—Although some polymerase α is bound to the matrix, the majority of polymerase α activity “leaks” from the nucleus to what is operationally defined as the “cytoplasm” in the process of isolating nuclei and the nuclear matrix (Foster and Collins, 1985). The subcellular localization of primase activity was found to differ markedly from that of polymerase α activity (Table II). Whereas only one-third of polymerase α activity remains bound to the nuclear matrix, over two-thirds of primase activity remains bound. To minimize the possibility of nonspecific binding of these proteins to the matrix, this experiment was repeated using matrices that had been subjected to extensive additional washing as described under “Experimental Procedures.” This treatment yielded only a 1% reduction in polymerase α activity and a 7% reduction in primase activity, indicating that the remaining polymerase α and primase activities are very tightly bound to the matrix.

Solubilization of Matrix Primase and Polymerase α—The solubilization of polymerase α and primase from the extensively washed matrix was accomplished by treatment with β-D-octylglucoside as described under “Experimental Procedures.” Total primase and polymerase α activity prior to solubilization was 58.78 pmol/h and 1.73 mmol/h, respectively.

**DISCUSSION**

In the present paper we have shown that, in contrast to HeLa matrix-bound polymerase α, which exhibits little enhancement of activity with the addition of exogenous template (Foster and Collins, 1985), a marked stimulation of matrix-bound primase activity was noted with the addition of poly(dT) (Fig. 1 and Table I). Differences in spatial orientation underlying this difference and their possible implications for replicative mechanisms remain to be explored. In contrast to polymerase α, primase in the HeLa cell is located predominantly bound to the nuclear matrix (Table II). This finding, that most of the primase in the cell exists in a bound form, partly explains the well-known difficulties in obtaining adequate amounts of soluble enzyme for analysis. In addition, we have described a method for the solubilization of primase and

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**Table I**

| Activity recovered% | Total | Soluble | Matrix |
|---------------------|-------|---------|--------|
| Primase             | 53.7  | 40.3    | 13.7   |
| Polymerase α        | 98.8  | 33.9    | 5.9    |

* Extensively washed nuclear matrix was treated with β-D-octylglucoside as described under “Experimental Procedures.” Total primase and polymerase α activity prior to solubilization was 58.78 pmol/h and 1.73 mmol/h, respectively.

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**Table II**

| % of total activity | Matrix | Soluble* |
|---------------------|--------|----------|
| Primase Washing     | 79.5%  | 20.5%    |
| Without             | 72.3%  | 77.7%    |
| With                | 33.3%  | 66.7%    |
| With                | 32.5%  | 67.5%    |

* Represents cytoplasmic and salt-extracted fractions.

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**Fig. 2.** Time course of the incorporation of [3H]AMP into the nuclear matrix. This reaction was carried out as described under “Experimental Procedures” using 30 μg of matrix protein. Values shown represent the mean of triplicate reactions.
polymerase α from the matrix with little loss of activity (Table III).

Since, in human systems, primase has successfully resisted separation from polymerase α (Wang et al., 1983, 1984), our data would seem to indicate that the majority of that polymerase α activity present in the form of a polymerase α-primase complex is also bound to the matrix. In accordance with this, we have recently noted in preliminary experiments with the enzymes solubilized from the nuclear matrix that >65% of the α activity was associated with primase (data not shown). Although the functional significance of the existence of multiple forms of polymerase α is unknown, it is conceivable that they serve an important replicative function. It has been suggested that the leading strand is replicated by the polymerase α devoid of primase activity, while the lagging strand, in frequent need of primer formation, is replicated by that polymerase α having associated primase (Ottiger and Hubscher, 1984). The importance of other enzymes reported to be present on the matrix, including topoisomerase (Nishizawa et al., 1984), remains to be determined. The ability to solubilize nuclear matrix replication complexes provides the potential to systematically explore these structural and functional relationships.

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