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Characterization of a factor that can prevent random transcription of cloned rDNA and its probable relationship to poly(ADP-ribose) polymerase

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ABSTRACT

A factor which eliminated nonspecific transcription of cloned rat rDNA was extensively purified from rat mammary adenocarcinoma ascites cells by successive fractionations on DEAE-Sephadex and heparin-Sepharose columns. The fractions containing RNA polymerase I (HS-B) and fractions eluting thereafter (HS-C) from the heparin-Sepharose column were pooled separately. Addition of HS-C to HS-B prevented random transcription of rDNA and yielded an accurate rDNA transcript with negligible non-specific transcription. The factor was essentially homogenous and corresponded to Poly(ADP-ribose) polymerase with respect to molecular weight, dependence on DNA for its activity and its ability to undergo auto ADP-ribosylation. The total amount of protein in the transcription assay was approximately 2 μg, which indicates a high degree of purity of all the factors required for specific transcription of rDNA.

INTRODUCTION

The components responsible for initiation of transcription must be characterized in order to elucidate the molecular mechanisms controlling intial transcriptional events. Using cell-free systems, factors essential for transcription by RNA polymerases II (1-3) and III (4-7) have been identified and in some cases, well characterized. On the other hand, progress in the characterization of specific factors involved in ribosomal gene transcription has been slow, probably due to instability of the potential regulatory factors. The dramatic arrest of rRNA synthesis *in vivo* by inhibitors of either mRNA (8,9) or protein (10-12) synthesis attests to the possibility that factors regulating initiation of rDNA transcription turn over rapidly (13-15). Elucidation of the nature of the specific components essential for rDNA transcription is a challenging problem particularly in view of the observation that transcription of rDNA from different species requires homologous fractions (16-18).

The enzymology, structure and the probable regulatory role of RNA polymerase I have been studied in our laboratory for a number of years (19-23). Recently, we have initiated studies on the identification and

characterization of factors involved in rDNA transcription using fractionated cell extract (24). Initially, an extract derived from rat adenocarcinoma ascites cells was fractionated successively by DEAE-Sephadex and heparin-Sepharose chromatography which resulted in a wash fraction (HS-A), and a fraction eluting with RNA polymerase I (HS-B). Neither fraction alone supports transcription but they do so following reconstitution. Subsequent studies demonstrated that if large quantities of cells were used for fractionation, HS-B fraction retains all the factors necessary for accurate transcription of rat rDNA (25). Although the major polypeptides in the HS-B fraction correspond to RNA polymerase I subunits, highly purified RNA polymerase I did not support accurate transcription of rDNA (25). These data indicate that other factors are essential for specific rDNA transcription. We have now purified one of the factors associated with the HS-B fraction. This factor, purified essentially to homogeneity, is functionally related to poly(ADP-ribose) polymerase and is analogous to the factor TFIIC that suppresses nick-induced transcription by RNA polymerase II (26).

MATERIALS AND METHODS

Fractionation of cell extract and purification of HS-C

Whole cell extract prepared from rat mammary adenocarcinoma ascites cells 6-7 days after transplantation, essentially as described by Manley *et al.* (27) was applied on a DEAE-Sephadex A-25 column equilibrated with Buffer A (20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic Acid, pH 7.9, 20% (v/v) glycerol, 0.1 mM EDTA and 2 mM dithiothreitol) containing 0.05 M $(NH_4)_2SO_4$. The column was washed with 0.05 M $(NH_4)_2SO_4$ in Buffer A and the bound proteins were eluted stepwise with 0.175 M $(NH_4)_2SO_4$ in Buffer A and 0.5 M $(NH_4)_2SO_4$ in Buffer A. Proteins eluted with 0.175 M $(NH_4)_2SO_4$ (DE-B) were further chromatographed on a heparin-Sepharose column. The latter column was equilibrated in Buffer B (50 mM Tris-HCl, pH 7.9, 20% (v/v) glycerol 0.1 mM EDTA, 5.0 mM $MgCl_2$ and 2 mM dithiothreitol) containing 0.2 M NH_4Cl. After application of DE-B to heparin-Sepharose, the column was washed with the same buffer (i.e. Buffer B containing 0.2 M NH_4Cl) and the bound proteins were eluted with a linear (0.2 $\sim$ 2.0 M) salt gradient. Proteins eluted with 0.5 M NH_4Cl (HS-B fraction) and at higher NH_4Cl concentrations (HS-C) were pooled separately, precipitated with $(NH_4)_2SO_4$ and dialysed against Buffer B (containing 50% v/v glycerol) prior to transcription assay.

Polyacrylamide gel electrophoresis under denaturing conditions

Aliquots of HS-C were precipitated with trichloroacetic acid (TCA) (10%

w/v final concentration) for 16h at 4°C. The precipitate was collected by centrifugation, washed once with 0.5 ml acetone and dissolved in 5 µl of 65 mM Tris-HCl (pH 7.0), 1% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol and 5% (v/v) β-mercaptoethanol. The sample and appropriate molecular weight markers were boiled for 2 minutes and subjected to electrophoresis using linear gradient (2-16%) polyacrylamide slab gels.

Preparation of nicked DNA

pBR322 containing the 2kb fragment of the ribosomal RNA gene (28) was nicked with DNAse I as described by Dynan and Burgess (29). The DNA was treated with the nuclease for 60 min at 15°C, phenol extracted and precipitated with ethanol.

Measurement of random transcription

Transcription assays contained 50 mM Tris-HCl, pH 7.9, 60 mM KCl, 5 mM $MgCl_2$ 0.1 mM EDTA, 10% (v/v) glycerol, 0.5 mM dithiothreitol, 500 µM of the three unlabeled ribonucleoside triphosphates, [α^{32}P]UTP, nicked DNA (DNAse I treated), 1.5 µg HS-B (which contains RNA polymerase I), 130 µg/ml α-amanitin to inhibit RNA polymerases II and III, and HS-C in a total volume of 25 µl. Samples were incubated for 30 min at 30°C and the reaction product was analyzed as described previously (30).

Specific transcription of rDNA

In vitro transcription assays were performed as described above except that cloned rat rDNA containing the initiation region was used as the template. The plasmids containing rat rDNA fragments (provided by Drs. Lawrence Rothblum and Christina Harrington) were used for transcription. A subclone that contains a 2.0 kb Sal 1 fragment of rDNA (Fig. 1A) was linearized with Xho 1 and the truncated template was used in a run-off transcription assay (28). Accurate transcription of this template must yield a 635 nucleotides long product (Fig. 1A) (25). The specific transcription of rDNA was assessed by running labelled markers of defined length in the same gel. The reaction was terminated by the addition of 50 mM Tris HCl (pH 8.0) containing 6 mM EDTA, 0.15M NaCl, 0.3M sodium acetate, 0.5% v/v SDS and 60 µg/ml tRNA. RNA was extracted with phenol, precipitated with ethanol and analyzed on 4% polyacrylamide gels in the presence of 7M urea. The gels were exposed to Kodak XAR-5 X-ray film at -70°C in the presence of an intensifying screen.

S_1 nuclease protection analysis

The 5'-termini of Sal 1 - Xho 1 rDNA fragment containing the initiation site was labelled with [γ^{32}P]ATP and was hybridized to the unlabelled RNA

synthesized from the Xho 1-cleaved plasmid DNA. After treatment with a single strand specific nuclease, RNA/DNA hybrid resistant to the nuclease was analyzed by the method of Berk and Sharp (31) as modified by Finansek et al. for the rat ribosomal RNA gene (32).

Incorporation of [^{14}C]NAD by HS-C fraction

HS-C fraction (1μg) was incubated at 37°C for 5 min in Tris-HCl buffer (pH 8.0) containing 10 mM $MgCl_2$, 1 mM dithiothreitol 10% (v/v) glycerol, nicked rDNA, 5 μCi/ml [^{14}C]NAD and 2.5 mM NAD. The reaction was terminated by the addition of 1% (v/v) SDS and the product analyzed by gel electrophoresis as described above. The gel was sliced (2 mm thick) and assayed for radioactivity after incubation with Protosol at 55°C for 2h.

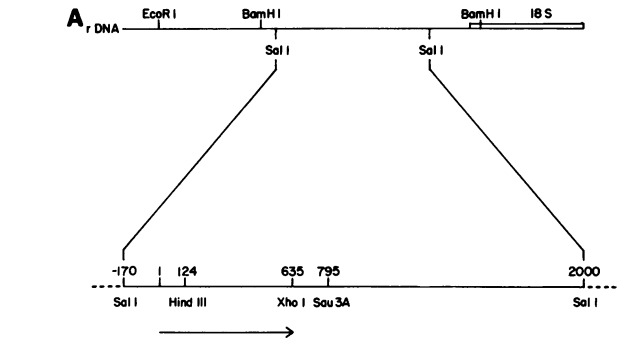
RESULTS

Molecular weight of the purified HS-C factor from whole cell extract

The cell extract was fractionated as described in the Methods section and is schematically represented in Figure 1B. The fraction eluting with 0.175 M $(NH_4)_2SO_4$ from DEAE-Sephadex column (DE-B fraction) was further chromatographed on a heparin-Sepharose column using a linear (0.2 m ⁓ 2 m) NH_4Cl. RNA polymerase I containing fractions eluting with about 0.5 M NH_4Cl (HS-B) and fractions eluting thereafter (> 0.5 M NH_4Cl; HS-C fraction) were pooled separately. When HS-C was subjected to SDS-PAGE, a major protein with M_r 116,000 was visible (Fig. 2) following staining with Coomassie blue.

Effect of HS-C factor on transcription of nicked rDNA

The nature of the HS-C factor was further investigated by using nicked DNA (see Methods) for transcription at varying concentrations of DNA and HS-C factor. α-amanitin was used at 130 μg/ml to inhibit RNA polymerases II and III. First, transcription using various amounts of nicked DNA ranging from 0.1-0.5 μg/25 μl of reaction volume was assayed in the absence or presence of a fixed concentration (1 μg/reaction) of HS-C. With 0.2 μg and 0.3 μg of DNA in the reaction, transcription in the presence of HS-C (as measured by incorporation of [α^{32}P]UTP into TCA-insoluble precipitate) was approximately 40% and 60%, respectively of the transcription obtained in the absence of HS-C (control). As the amount of the template increased, incorporation of [α^{32}P]UTP into the acid-insoluble precipitate increased, suggesting an increase in the random transcription at a lower HS-C/DNA ratio (Fig. 3a). Thus, when 0.5 μg of the template was used for transcription, the incorporation of radioactivity into acid-insoluble precipitate was about 70% of that observed in the absence of HS-C. These data suggest that HS-C becomes



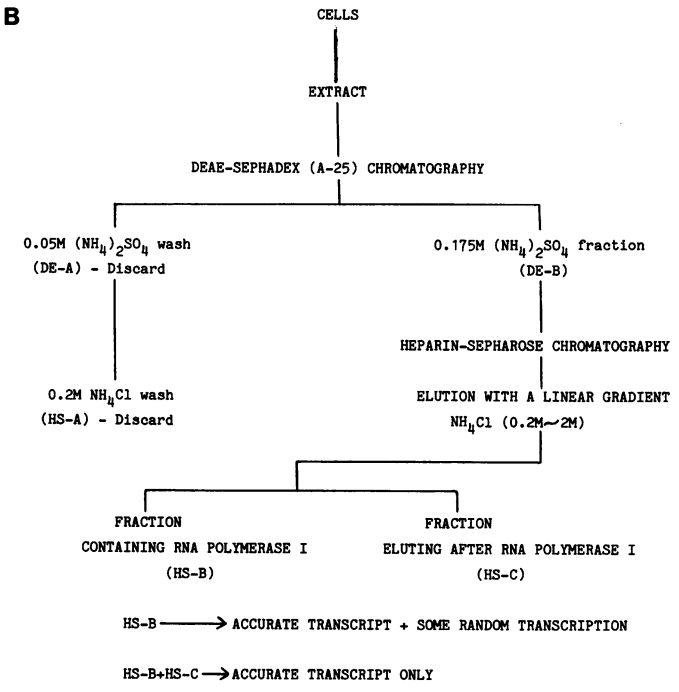


Fig. 1A. The Sal I fragment of rat rDNA containing the transcription initiation site cloned in pBR322. The size of run-off transcripts generated in vitro with the cloned fragment when cleaved with the indicated restriction enzymes is shown. Linearization with Xho 1 will yield a 635 nucleotide long transcript in vitro, if transcription is initiated at nucleotide +1 (indicated by the arrow). For the present studies, the Xho 1-cleaved template was used.
Fig. 1B. Scheme for fractionation of whole cell extract.

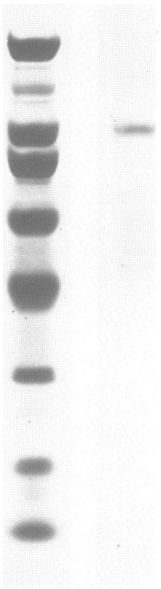


Fig. 2. Electrophoresis of HS-C fraction under denaturing conditions. Proteins (2 μg) were precipitated with trichloroacetic acid, washed with acetone and subjected to SDS-PAGE using linear polyacrylamide gradient (2- 16%) slab gels. Molecular weight of HS-C fraction (right lane) was estimated from markers run on the same gel (left lane). The marker that ran almost parallel to the factor corresponds to β-galactosidase (M_r 115,000).

a limiting factor at higher concentrations of nicked DNA thus allowing the random transcription to continue at a rate close to the control level. However, when unnicked DNA was used as template for random transcription, the incorporation of [α-^{32}P]UTP was lower than that obtained with same amount of nicked DNA (data not shown).

The ability of the HS-C factor to inhibit random transcription was also studied using various amounts of HS-C at a fixed DNA concentration. Four concentrations of HS-C ranging from 0.2 to 1.0 μg/25 μl of reaction volume were used with 0.2 μg of DNA (Figure 3b). Consistent with the data presented in Figure 3a, the incorporation of [α^{32}P]UTP into TCA-insoluble precipitate decreased with increasing concentrations of HS-C factor. With 0.2μg of HS-C in the reaction, transcription was 80% of that observed in its absence. Increasing the HS-C concentrations decreased the transcriptional activity proportionally. At the highest concentration of HS-C (1 μg/assay), transcription was inhibited by as much as 60% of the control.

At a fixed concentration of HS-C, inhibition of overall transcription with nicked DNA was 49% as opposed to 29% with control DNA (data not presented). In one experiment, inhibition of transcription using nicked DNA was as much as 60%. These differences in the activity of HS-C are most

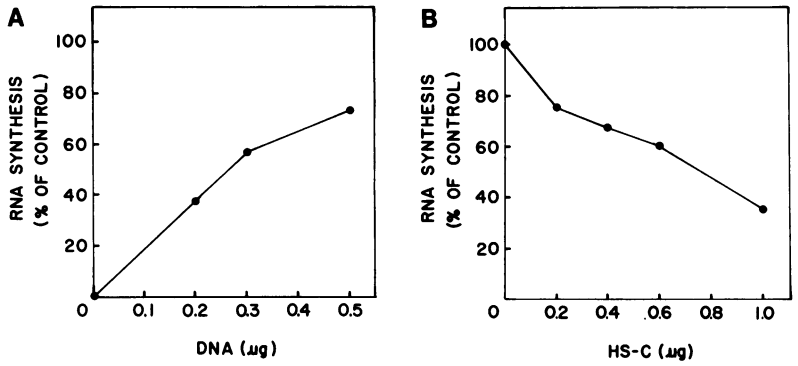


Fig. 3A. Effect of HS-C on random transcription of varying amounts of nick-induced rDNA. Transcription using varying amounts of nicked rDNA was measured in the presence of constant amount of HS-C (1 µg) and α-aminitin (130 µg/ml) to inhibit RNA polymerases II and III as described in the Methods.

Fig. 3B. Inhibition of nick-induced transcription of rDNA by different amounts of HS-C. rDNA (0.2 µg) which had been nicked with DNAse I was assayed for random transcription in the presence of increasing amounts of HS-C as described in the Methods.

probably due to varying number of nicks in the DNA preparations. In any case, the effect of HS-C was less evident on untreated DNA preparations.

Probable relationship of HS-C factor to poly[ADP-ribose]polymerase

The activity of HS-C was dependent on nicked DNA, which is an established property of purified poly (ADP-ribose) polymerase (33,34). The binding of the enzyme to double-stranded DNA appears to be a prerequisite for its catalytic activity (34). It is also known that poly[ADP-ribose]polymerase by itself can be ADP-ribosylated (34). The similarity in the molecular weight of HS-C with that reported for authentic poly[ADP-ribose] polymerase (33) prompted us to investigate if HS-C can undergo auto ADP-ribosylation. To test this possibility, HS-C was incubated with [^{14}C]NAD in the presence of nicked DNA at 37^{o}C for 5 min. The reaction was terminated by the addition of SDS and subjected to electrophoresis on a linear gradient (2-16%) slab gel. The gel was then sliced and radioactivity measured. As shown in Fig. 4, most of the radioactivity was found either near the origin (top of the gel) or at a position that approximately corresponded to the Coomassie blue-stained band obtained with HS-C fraction run in a parallel gel track. These two radioactive peaks should correspond to the different levels of ADP-ribosylation of the enzyme which is known to alter the electrophoretic mobility of authentic poly (ADP-ribose) polymerase (35). When HS-C fraction was incubated with [^{14}C]NAD at 4^{o}C, no incorporation of [^{14}C]NAD was observed

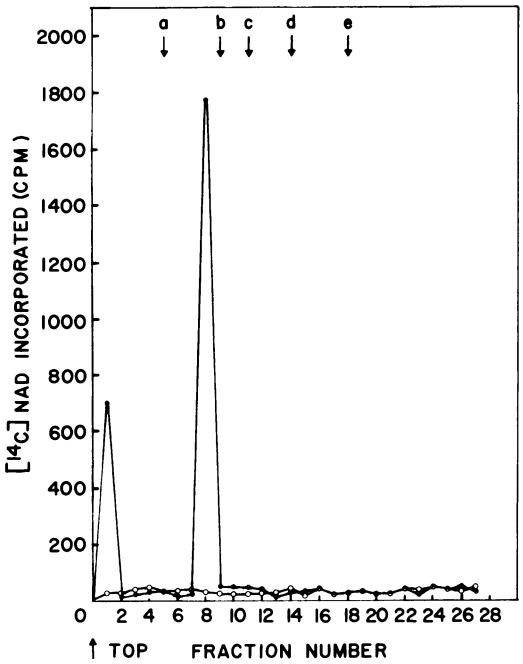


Fig. 4. ADP-ribosylation of HS-C. HS-C (1 µg) was incubated with [^{14}C]NAD as described under Methods either at 37°C (●) or at 4°C (o) for 5 min. Reactions were terminated by the addition of SDS followed by β-mercaphethanol, the tubes were heated to 100° C for 2 min and the proteins electrophoresed on a linear gradient (2-16%) slab gels. The gels were sliced (2 mm thick) and assayed for radioactivity. HS-C run in a parallel lane, stained with Coomassie blue and sliced, was present in fraction 8. The positions of molecular weight markers-myosin, β-galactosidase, phosphorylase B, bovine serum albumin and ovalbumin are indicated by a through e.

indicating that the effect of the HS-C fraction is due to a specific enzymatic reaction. Similarly, in the absence of DNA, there was no detectable incorporation of [^{14}C]NAD into trichloroacetic acid-insoluble precipitate (data not presented).

Specific transcription of cloned rat rDNA in the presence of HS-C

To determine if the HS-C fraction can promote specific transcription of rDNA by preventing random transcription, we used Xho 1-cleaved rat rDNA in a run-off transcription assay (see Materials and Methods and Fig. 1A). With HS-B alone, the anticipated product (635 nucleotides in length) was obtained (Fig. 5). However, the clarity of the transcript was masked by random transcription (Fig. 5, lane b). When HS-C was included in the reaction, there was a dramatic reduction in the non-specific transcription. The inhibition of random transcription by HS-C was concentration dependent. As little as 0.6 µg of HS-C was sufficient to obliterate most of the random transcription. Increasing the concentration of HS-C to 0.8 µg virtually abolished random transcription. Higher concentrations of HS-C (1.0 µg) not only inhibited random transcription but also diminished the intensity of the accurate transcript (Fig. 5 lanes c,d,e). The effect of HS-C was so profound that in the presence of HS-C, the autoradiogram was changed from a dark smear to a single well-defined band which corresponds to a size of 635 nucleotides. A

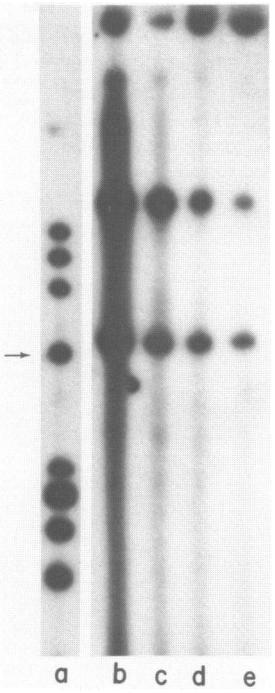


Fig. 5. Specific transcription of rDNA in the presence of HS-C. Cloned rDNA was transcribed using HS-B as detailed under Methods in the absence (lane b) or presence of HS-C (lane c 0.6 μg) lane d (0.8 μg) lane e (1.0 μg). The arrow indicates the position of the 603 base pair fragment of ^{32}P-labelled ϕ X174 RF DNA used as a molecular weight marker (lane a).

transcript of higher size observed routinely was due to a DNA-independent reaction; it was resistant to actinomycin D or DNase. Interestingly, the radioactivity associated with this band was also reduced by higher concentration of HS-C. It is obvious that a critical concentration of HS-C is needed to achieve maximal accurate transcription. No distinct transcripts were obtained when rDNA nicked with DNase was used as the template even in the presence of high levels of HS-C (data not presented). Clearly, if the nicking of DNA exceeds a limit it would be technically difficult to obtain the correct HS-C/DNA ratio and optimum amount of transcriptionally active RNA polymerase I and other factors for achieving specific transcription.

S_1 nuclease analysis

The accuracy of the *in vitro* initiation site was confirmed by the S_1 nuclease protection assay. RNA transcribed *in vitro* was hybridized to 5'-labelled Sal 1-Xho 1 rDNA fragment. After digestion with S_1 nuclease, the protected DNA was analyzed by gel electrophoresis (see Materials and Methods). As shown in Fig. 6, a single protected band corresponding to a 635 nucleotide long fragment was observed. This data further proves that the 635 nucleotide long transcript was indeed transcribed from the nucleotide + 1 of rDNA.

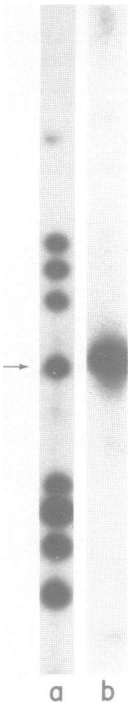


Fig. 6. S_1 nuclease mapping of *in vitro* synthesized RNA. RNA synthesized *in vitro* using HS-B (1.5 µg), HS-C (0.8 µg) and Xho 1 cleaved template, was hybridized to 5'-labelled Sal I-Xho 1 fragment, treated with S_1 nuclease, denatured and analyzed by gel electrophoresis (lane b). X174 RF DNA Hae 111 fragments labelled with γ^{32}P-ATP and T_4 polynucleotide kinase used as molecular weight standards (lane a).

DISCUSSION

The present studies have achieved purification of a factor that can prevent random transcription of rDNA by the RNA polymerase I-enriched fraction. Since the specific transcript is not degraded to smaller products (Fig. 5), the general decrease in transcription by HS-C (Fig. 3b) must not be due to RNAse activity that may be associated with HS-C. Moreover, almost all the RNAse activity is known to be eluted in the wash fraction (DE-A fraction, see Fig. 1) of the DEAE-Sephadex column (36). The total amount of protein necessary to achieve specific transcription of rDNA is only 1-2 µg as compared to 45 µg of protein required for achieving comparable transcription with whole cell extract. To our knowledge, this is the highest purification of a factor that can support accurate transcription of rDNA by preventing nonspecific transcription. Although fractions which transcribe rDNA accurately have been separated by phosphocellulose (PC) chromatography (37,38), these fractions have not been purified. Further, none of the factors regulating RNA polymerase I-directed transcription have been characterized. One of the

fractions (PC-B, eluting at 0.4 M salt) appears to contain an activity that eliminates random transcription of mouse rDNA (37). However, Poly (ADP-ribose) polymerase which has been well characterized is eluted at 0.6 M salt from the PC column (26). Thus, the relationship of the impure PC-B fraction to poly (ADP-ribose) polymerase is not evident.

The HS-C factor purified by us probably corresponds to poly(ADP-ribose) polymerase for the following reasons: First, its reaction is dependent on DNA, a unique property of poly(ADP-ribose) polymerase (34). Second, its molecular weight is similar to that reported for highly purified poly(ADP-ribose) polymerase from calf thymus (39) and HeLa cells (40) and third, it can undergo ADP-ribosylation by itself, most heavily ADP ribosylated form having the least mobility on SDS-polyacrylamide gel (Fig. 4) as observed with authentic poly(ADP-ribose) polymerase (35).

The ability of HS-C to inhibit random transcription obtained with a defined template like the cloned rat rDNA suggests that the DNA may be nicked. That nicking of DNA is obligatory to the action of HS-C is further evidenced by the fact that the extent of inhibition of overall transcription is higher with nick-induced DNA than with DNA preparation that contains fewer nicks (see RESULTS). Nicking in the latter preparation may be caused during isolation of plasmid DNA using the polyethylene glycol method. This method involves separation of plasmid DNA from RNA at low temperatures [-8°C to -10°C]. Freezing of the mixture during this step could inevitably lead to some nicks in the DNA. Alternatively, DNA might be nicked during *in vitro* transcription by traces of DNAses still present in the HS-B fraction. In either case, removal of HS-C factor will result in exposure of the "induced" nicks in the template to RNA polymerase I. DNA strand breaks can also be induced *in vivo* by agents including carcinogens that result in activation of poly (ADP-ribose) polymerase (for review, see ref. 41). The increased synthesis of poly (ADP-ribose) is in some way involved in DNA repair (42,43) although the exact mechanism whereby DNA repair is brought about by poly (ADP-ribose) polymerase has not been elucidated. It thus seems plausible that HS-C has an important physiological role *in vivo* to prevent nicking in DNA caused by activation of certain nucleases or by exogenous agents.

Recently, Slattery *et al.* (26) demonstrated that the factor TF11C which eliminates random transcription by RNA polymerase II is identical to poly(ADP-ribose) polymerase. This factor was purified from HeLa cells by successive chromatographic fractionation on phosphocellulose (1 M KCl fraction), DEAE-cellulose (0.05 M KCl breakthrough fractions) and DNA-

cellulose (0.6 M KCl fraction) columns. Although we used a different protocol for purification of factors required for transcription by RNA polymerase I, the final preparation has properties very similar to those reported for TFIIC.

Finally, the fractionation of the factor on heparin-Sepharose column deserves some comment. In experiments using relatively large quantities of cells (> 1 x 10^9) for preparation of extract, significant amount of HS-C factor remained bound to HS-B fraction. Consequently, one can obtain specific transcription of rDNA without further addition of HS-C. Another factor contributing to the separation of HS-B and HS-C is the use of freshly prepared heparin-Sepharose column. Accordingly, we have routinely employed such columns for dissociation of HS-C from HS-B. Even the HS-B fraction prepared in this manner is likely to contain some HS-C factor. In fact, increasing the amount of HS-B fraction as much as 10-fold used in the transcription assay can eliminate some random transcription; but even at this concentration of HS-B, random transcription cannot be completely eliminated unless HS-C is added to the system (data not shown). Thus, specific transcription of rDNA by HS-B fraction is achieved only by obtaining this fraction together with HS-C or by adding exogenous HS-C to HS-B from which the majority of the HS-C factor has been removed. We are now in the process of characterizing additional transcriptional factors that constitute the HS-B protein complex.

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