Faithful transcription of human mitochondrial DNA has been reproduced in vitro, using a fraction of mitochondrial proteins capable of accurate initiation at both the heavy- and light-strand promoters. Here we report the initial dissection of this system into two nonfunctional components which, upon mixing, reconstitute promoter-specific transcriptional capacity in vitro. One of these components copurifies with the major nonspecific RNA polymerase activity, suggesting its identity. The other component lacks significant polymerase activity, but contains a protein or proteins required for accurate initiation at the two individual promoters by isolated mitochondrial RNA polymerase. This factor facilitates specific transcription, but has little or no effect on nonspecific transcription of a synthetic copolymer (poly(dA-dT)), indicating a positive role in proper promoter recognition. The transcription factor markedly stimulates light-strand transcription, but only moderately enhances transcription initiation at the heavy-strand promoter, suggesting different or additional factor requirements for heavy-strand transcription. These requirements may reflect the functional differences between heavy- and light-strand transcription in vivo and, in particular, the role of the light-strand promoter in priming of heavy-strand DNA replication.

Complete nucleotide sequences are known for mouse (1), human (2), and bovine (3) mtDNA. The overall mode of transcription of these genomes is clearly a reflection of their unique organization. The striking compactness of mammalian mtDNA, with little or no intragenic spacer sequences throughout much of its length, places constraints on the number, size, and situation of regulatory elements for both transcription and replication (4). Indeed, with the exception of the light-strand (L-strand) origin of replication, all control elements identified thus far have been found in the displacement-loop (D-loop) region. These include major promoters for both heavy (H)- and L-strand transcription, as well as the origin of replication for the H-strand. Once initiated in the D-loop, transcription proceeds symmetrically around the circular genome, producing polycistronic precursor RNAs encompassing most, if not all, of the genetic information potentially encoded. Maturation of these precursors to mRNAs, tRNAs, and rRNAs requires multipleprecise processing events.

The isolation, from human KB cell mitochondria, of a protein fraction capable of initiating transcription accurately on a cloned human D-loop template (5) has made possible in vitro analyses of the sequence-specific protein-nucleic acid interactions required for this event. Through the use of this fraction and a series of D-loop deletion mutants constructed in vitro, the major promoters for both H- and L-strand transcription (HSP and LSP, respectively) were delimited to small regions surrounding the start sites (6, 7). Further analysis by site-directed mutagenesis has identified nucleotides within the promoters critical for their in vitro function (8). While these two promoters are similar to one another, they bear no resemblance to any sequence found near the 5' ends of mouse in vivo D-loop strands (9).

This partially purified RNA polymerase activity has allowed us to define in vitro correlates of in vivo transcriptional events occurring in KB cell mitochondria and to probe their DNA sequence requirements. Here we report the chromatographic resolution of mtRNA polymerase activity and a transcription factor required for accurate initiation at both the HSP and LSP. Following chromatography on phosphocellulose, the fraction containing the peak of RNA polymerase active on a synthetic, nonspecific template shows only very weak selectivity for the HSP or the LSP in run-off assays. When the polymerase is supplemented with a fraction eluted at higher ionic strength, specific initiation is restored. We demonstrate that this fraction contains a protein factor (or factors) that acts as a positive regulator of mitochondrial transcription.

**EXPERIMENTAL PROCEDURES**

Initial RNA Polymerase Preparation—A heparin-Sepharose mtRNA polymerase fraction was prepared essentially as described by Walberg and Clayton (5), with the following modifications. Mitochondria isolated from human KB cells in late logarithmic growth phase by the method of Bogenhagen and Clayton (10) were centrifuged in a 1.0–1.5 M sucrose step gradient, collected, and diluted 2-fold with mitochondrial storage buffer (20 mM Tris-Cl, pH 8.0, 0.5 mM EDTA, 0.25 M sucrose, 15% (v/v) glycerol). Dithiothreitol (DTT) was added to a final concentration of 1 mM, and the mitochondria were centrifuged in a JA-20 rotor for 15 min at 15,000 rpm (18,000 × g). After the supernatant had been decanted, the pellet was frozen in liquid nitrogen and stored at −70 °C. Frozen mitochondria from 24 liters of KB cells were thawed on ice, washed once with 100 ml of 20 mM Tris-Cl, pH 8.0, 0.2 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 0.25 M sucrose, 15% glycerol, and pelleted.
Detergent lysis and heparin-Sepharose chromatography were carried out as described (6), except that centrifugation to clear the lysate was at 45,000 rpm for 60 min in a Type 75Ti rotor (131,000 × g), MgCl₂ was omitted from all buffers and the EDTA concentration was 0.1 mM; 0.1% (v/v) Triton X-100 was included throughout the chromatography, and the phenylmethylsulfonyl fluoride concentration was 0.5 mM. Finally, all reported volumes were increased 4-fold.

Phosphocellulose Chromatography—After active heparin-Sepharose fractions had been pooled, one-fourth volume (4 ml) was dialyzed against enzyme storage buffer (10 mM Tris-Cl, pH 8.0, 0.1 mM EDTA, 1 mM DTT; 0.1% (v/v) Triton X-100 was included throughout the chromatography). The column was washed with the same buffer (1-2 column volumes) until a constant A₅₄₀ was recorded, and this step was repeated with column buffer plus 300 mM KC1. The column was equilibrated with a linear 0.3-0.8 M KC1 gradient in column buffer at a flow rate of 20 ml/h. Twenty 6-ml fractions were collected and assayed for KC1 concentration by conductivity and for protein concentration by the method of Schaffner and Weissman (11).

The heparin-Sepharose pool of mitochondrial RNA polymerase activity was assayed with the synthetic alternating copolymer poly(dA·dT) as template. Standard reaction conditions contained 10 mM Tris-Cl, pH 8.0, 10 mM MgCl₂, 1 mM EDTA, 100 µg/ml RNase-free BSA, 400 µM ATP, 150 µM CTP, 150 µM GTP, 0.2 µM [α-³²P]UTP (specific activity 410 Ci/mmol) at 28 °C for 30 min, after which nucleic acids were precipitated in 2 ml of 5% trichloroacetic acid, 50 mM sodium pyrophosphate, and filtered through Whatman GF/B paper, and incorporation of radioactivity was assayed by liquid scintillation spectroscopy. Except where noted, 4 µl of dialyzed enzyme fractions were assayed in 25-µl total volume; thus, the final glycerol and Triton X-100 concentrations were 8 and 0.016%, respectively. Specific activity determinations were performed at a UTP concentration of 10 µM (in 50-µl total volume) with all other parameters as above. One unit of poly(dA·dT)-directed RNA polymerase activity is defined as the incorporation of 1 pmol of UMP into cold-insoluble sediment in 30 min under these reaction conditions.

In Vitro Transcription Assays—Non-specific RNA polymerase was assayed with the synthetic alternating copolymer poly(dA·dT) as template. Standard reaction conditions contained 10 mM Tris-Cl, pH 8.0, 10 mM MgCl₂, 1 mM EDTA. The column was washed with the same buffer (1-2 column volumes) until a constant A₅₄₀ was recorded, and this step was repeated with column buffer plus 300 mM KC1. The column was equilibrated with a linear 0.3-0.8 M KC1 gradient in column buffer at a flow rate of 20 ml/h. Twenty 6-ml fractions were collected and assayed for KC1 concentration by conductivity and for protein concentration by the method of Schaffner and Weissman (11).

One-half volume of each fraction was then dialyzed against enzyme storage buffer and stored at −20 °C, and the remainder was frozen in liquid N₂ and stored at −70 °C. Dialyzed fractions were used in all transcription assays.

**RESULTS**

**Isolation of a Non-specific mtRNA Polymerase**

The heparin-Sepharose pool of mitochondrial RNA polymerase activity from human KB cells is capable of initiating transcription accurately at both the H- and L-strand template for cloned human mtDNA, shown in linearized restriction fragments (5-8). The synthetic alternating copolymer poly(dA·dT) can also serve as an in vitro template for the HS pool activity.

**Reconstitution of Specific Transcription**

Two possible circumstances could account for this discrepancy between specific and non-specific activity profiles. One is nonidentity of the two types of activity, indicating the presence of two (or more) RNA polymerases, one intrinsically specific, the other not, in the HS pool (PC load). Alternatively, accurate initiation at the LSP and elongation of the full-length run-off transcript might depend on two or more dis-
Human Mitochondrial Transcription

possibilities. Fig. 3 displays the RNA species produced by PC fractions 15-20 in the absence (−) or presence (+) of poly(dA-dT)-directed RNA polymerase peak fraction (mt RNAP, assayed alone in the leftmost lane). Marked enhancement of LSP-specific transcription is clearly dependent on the added polymerase, with a peak in fraction 17 (hereafter termed “mitochondrial transcription factor”); eluted at 0.64 M KCl). The complementary mixing experiment, shown in Fig. 2B, demonstrates copurification of nonspecific polymerase with the factor-dependent promoter-directed polymerase activity in PC fraction 10. The data suggest that an intrinsically nonselective mtRNA polymerase is necessary, but not sufficient for LSP-specific transcription.

Characterization of Mitochondrial Transcription Factor

The enhancement effect of mixing transcription factor and polymerase fractions is not simply additive, as the titrations shown in Fig. 4 clearly demonstrate. The fluorographed gel in Fig. 4A shows increasing amounts of transcription factor assayed for run-off activity in the absence (lanes A-D) and presence (0.44 and 0.73 poly(dA-dT) units: lanes E-I and J-N, respectively) of mtRNA polymerase. The species at ~280 nucleotides is a pCp-labeled RNA fragment added to the

Fig. 2. Assay of human mtRNA polymerase chromatographed on phosphocellulose. A, phosphocellulose fractions were assayed for poly(dA-dT)-directed incorporation of [α-32P]UMP, protein concentration (O), and KCl concentration (---), as described under “Experimental Procedures.” The peak of RNA polymerase activity is seen in fraction 10, eluted at 0.45 M KCl. B, phosphocellulose fractions 7-12 (4 μl each) were assayed for LSP-directed run-off transcription in the absence (−) or presence (+) of mitochondrial transcription factor (mt TF). Transcription factor alone is assayed in the leftmost lane. The template was EcoRI-digested pKB741SP (16 μg/ml), and the length of the expected run-off product initiated at the LSP is 416 nucleotides (arrowhead). Transcription factor, or a compensating buffer (2 μl), was added to the reaction mixture prior to the addition of RNA polymerase, but no preincubation was required. The final glycerol and Triton X-100 concentrations in these experiments were 12 and 0.024%, respectively. All other conditions were as described under “Experimental Procedures.” Size estimates of the products were based on the migration of 32P-labeled HpaI-digested pBR322 DNA.

FIG. 3. Assay of mitochondrial transcription factor chromatographed on phosphocellulose. Phosphocellulose fractions 15-20 (1 μl each) were assayed for run-off transcription initiating at the LSP in the absence (−) or presence (+) of 1.2 poly(dA-dT) units of mtRNA polymerase (PC fraction 10), assayed alone in the leftmost lane. Reaction conditions are identical to those in the experiment of Fig. 2B, except that the final glycerol and Triton X-100 conditions were 10 and 0.02%, respectively. mt RNAP, mitochondrial RNA polymerase.
transcription mixture before distribution among individual reactions. Quantitative recovery of this undegraded species from all reactions excludes nuclease inhibition as a significant component of transcription factor action. In Fig. 4B, the factor effect on LSP-specific transcription is quantitated. Fluorograms were scanned densitometrically as described in the legend to Fig. 4, with specific transcription defined as the ratio of the intensity of the run-off signal at –280 nucleotides to that of the internal control band in each lane. At low levels of transcription factor, specific transcription is seen when the two fractions are present simultaneously. In contrast, a dramatic stimulation of poly(dA-dT)-directed [32P]UMP incorporation by mtRNA polymerase to increasing amounts of transcription factor concentration, which does not seem to depend on the amount of polymerase activity, as seen in Fig. 5.

Characterization of mtRNA Polymerase

Cation Response—Walberg and Clayton (5) characterized the HS pool RNA polymerase activity with respect to salt optima and divalent cation requirements, using denatured calf thymus DNA as template. Fig. 6A (closed circles) shows the response of PC RNA polymerase activity, directed by poly(dA-dT), to KCl concentration. The drastic inhibition even in low salt concentrations is characteristic of mtRNA polymerases from human (6), Xenopus (13), and yeast (14), when programmed with nonspecific templates. Fig. 6B shows that LSP-specific transcription by the PC enzyme, supplemented with transcription factor, responds very similarly to ionic strength. The open circles in Fig. 6A represent a plot of densitometric scanning data obtained from this gel.

Purification and Yield—Purification data for mtRNA polymerase are summarized in Table I. The nonspecific assay, with the synthetic poly(dA-dT) template, was carried out with 10 μM UTP present to quantitate RNA polymerase activity in crude lysate, HS pool, and PC fractions. In a separate experiment (not shown), all three fractions were shown to have a linear time course of incorporation for at least 40 min with the poly(dA-dT) template. The crude detergent-high salt lysate, cleared by centrifugation (S-130), supports very low incorporation with the synthetic template, but is extremely active in the run-off assay with EcoRI-digested pKB741SP (Fig. 7, lanes A–C), allowing the alternative estimates of purification and yield presented in Table II. Agreement between our two comparisons of HS pool and PC RNA polymerase, using either poly(dA-dT) or the mtDNA LSP, strengthens the argument for identity between the nonspecific and specific polymerase activities. Moreover, this equivalence of the specific to nonspecific activity ratios in HS pool and the PC system strongly suggests that the reconstitution of the LSP-specific activity after PC fractionation is complete, i.e.,

![Fig. 4. Quantitation of transcription factor activity. A, increasing amounts (0.3, 0.9, 1.5, and 2.4 μl) of mitochondrial transcription factor (PC fraction 17) were assayed for LSP-selective activity without added mtRNA polymerase (lanes A–D), with 0.44 poly(dA-dT) units (lanes E–I), and with 0.73 units (lanes J–N) of mtRNA polymerase. Lanes E and J show 0.44 and 0.73 units, respectively, of polymerase assayed without added transcription factor. The band (open arrowhead) migrating ahead of the run-off band (closed arrowhead) is a pCp-labeled RNA fragment (gift of D. D. Chang of this laboratory) added to the transcription mixture prior to distribution to individual reactions and used in gel scanning as an internal control for lane width, recovery, and nuclease activity. An incubation lacking any mitochondrial proteins is shown in lane O. Reaction conditions are as above, but with final glycerol and Triton X-100 concentrations of 11 and 0.022%, respectively. B, the fluorographed gel in A was scanned densitometrically, using a Beckman DU-8 spectrophotometer with scanning attachment. Specific transcription is defined as the ratio of intensity of the run-off signal at –416 nucleotides to that of the internal control band at ~280 nucleotides. It is plotted as a function of increasing mitochondrial (mt) transcription factor in the absence of mtRNA polymerase (○), and in the presence of 0.44 units (○) and 0.73 units (A) of mtRNA polymerase. C, response of poly(dA-dT)-directed [32P]UMP incorporation by mtRNA polymerase to increasing amounts of transcription factor. Transcription factor (0–2.4 μl) was added to reactions containing zero (○), 0.44 (○), and 0.73 (A) units of mtRNA polymerase, and incorporation was measured as described under “Experimental Procedures” (final glycerol and Triton X-100 concentrations: 9 and 0.018%, respectively).
FIG. 5. Heat lability of transcription factor activity. PC fraction 17, containing the peak of mitochondrial transcription factor, or PC fraction 10, containing the mtRNA polymerase, was heated at 100 °C for 5 min and then allowed to cool to room temperature. Run-off transcriptions were then carried out as follows: untreated RNA polymerase only (lane A), untreated RNA transcription factor only (lane B), untreated factor + untreated polymerase (lane C), heated factor + untreated polymerase (lane D), and untreated factor + heated polymerase (lane E). Final glycerol and Triton X-100 concentrations were 12 and 0.024%, respectively.

The striking discrepancy between activities measured for S-130 in the two assays can be rationalized in two, not mutually exclusive, ways. On the one hand, inhibition of nonspecific transcription due to DNA-binding proteins, to activities, such as ATPases and kinases, which could compete for substrates, and perhaps to nucleic acids might be expected in such a crude fraction. The greater than quantitative recovery of poly(dA-dT) RNA polymerase activity after heparin-Sepharose chromatography probably reflects, at least in part, the removal of such interfering or competing activities. It is noteworthy, however, that contaminating nucleases, active either on the double-stranded DNA template or on RNA products, do not seem to interfere, at least with run-off activity, as judged by the comparable signal-to-background ratios and internal control recovery at all three stages of purification (Fig. 7). However, nucleases or binding proteins with a preference for single-stranded DNA could skew the results in the direction observed, since the poly(dA-dT) template might be predicted to exist largely in single-stranded form at the low ionic strength of the transcription reaction. On the other hand, such a dramatic difference—in effect, an increase in selectivity on the order of 100-fold relative to HS pool—suggests the presence of specific stimulatory factors in the S-130. These could act either by suppressing nonspecific initiation preferentially (i.e., inhibiting a competing reaction) that no additional specificity factors are present in the HS pool.

The low yield of LSP-specific activity after heparin-Sepharose chromatography (Table II) might actually reflect loss of such factors, analogous to the dissociation of transcription factor on phosphocellulose. More recently, we have detected an additional activity (or activities) capable of selectively stim-

Table I

| Purification of nonspecific human mtRNA polymerase |
|---------------------------------------------------|
| Fraction                                      | Protein | Specific activity | Yield |
|------------------------------------------------|---------|------------------|-------|
| Cleared lysate (S-130)                        | 36,931  | 0.14             | 132   |
| Heparin-Sepharose pool                        | 486     | 13.9             | 10.5  |
| Phosphocellulose peak                         | 17.1    | 41.6             |       |

*a One unit = 1 pmol of UMP incorporated in 30 min with poly(dA-dT) template.

*b Relative to immediately preceding stage.
was omitted from the quantitation of "specific transcription units" radioactive nucleotide is \([\alpha^{32}\text{P}]\text{UTP}\); transcription conditions were supplemented with 2 µl of mitochondrial transcription factor. 

was 14 and 0.028%, respectively.

loss is evident in increasing amounts of the cleared lysate (S-130): HS, S-130, 18.0, 130

Fraction Specific Yield Selectivity
units/µg % LSP units/poly(dA-dT) units
S-130 18.0 100 LSP 130
HS pool 14.0 100 LSP 101
PC peak 40.1 101 LSP 0.96

One unit of LSP-specific activity = the amount of run-off product formation catalyzed by 1 unit of poly(dA-dT)-directed RNA polymerase activity in the experiment shown in Fig. 7 (extrapolated value derived from densitometric data).

Relative to immediately preceding stage.

ulating specific transcription from both the HSP and LSP by fractionation of a cleared lysate on DEAE-Sepharose. A fraction which flows through this resin at 0.1 M KCl contains no independent polymerase activity (specific or nonspecific), but can stimulate specific transcription in a HS pool or a PC-fractionated, reconstituted system by at least 40-fold. Moreover, promoter-specific stimulation of PC-purified RNA polymerase by this flow-through fraction is dependent upon added transcription factor.2

When poly(dA-dT)-directed transcription is used to calculate the RNA polymerase yields (Table I), the heparin-Sepharose step appears to be very efficient. Although major losses early in the purification cannot be ruled out, we have consistently failed to detect RNA polymerase activity (specific or nonspecific) in heparin-Sepharose flow-through fractions (data not shown). Inactivation of the enzyme may occur during heparin-Sepharose fractionation, but seems unlikely in light of the stability to storage at −20 °C of RNA polymerase activity at all stages of purification (at least 3 months for HS and PC, at least 1 month for S-130). Losses due to nonspecific adsorption or dilution, furthermore, would be predicted to be greater during subsequent dialysis and phosphocellulose chromatography steps, where the total protein concentrations are much lower.

The poor yield of RNA polymerase from phosphocellulose chromatography can be attributed almost entirely to losses during the final dialysis against 50% glycerol for storage and subsequent in vitro analysis. A greater than 3-fold reduction in volume is achieved, without changing the measured protein concentration (compare Table I with Fig. 2A). Thus, without the addition of carrier protein, roughly 70% of the protein in the PC peak fraction is lost by adsorption to the dialysis tubing. After correcting for this loss, the actual yield is closer to 75%, including PC peak fraction 10 and side fractions 9 and 11.

Transcription Factor Activity at the HSP

At the high DNA concentrations used to assay LSP-specific transcription, little or no HSP-specific run-off product was expected3 or observed, even with S-130 and HS pool enzymes. Moreover, reliable detection of the full-length HSP run-off product at −19 nucleotides is made problematic by LSP-dependent products of similar size (see above). However, when compared at low concentrations of templates lacking the LSP, roughly equivalent low levels of specific H-strand transcription are seen in the PC-fractionated, reconstituted system and in the HS pool. In lanes A–C of Fig. 8A, the HSP-specific run-off activity of the HS pool is assayed at 0.25, 1.0, and 4.0 µg/ml DNA. An identical titration was performed using an equivalent amount of PC-purified RNA polymerase activity (equal units of poly(dA-dT)-directed activity) in the presence (lanes E–G) or absence (lanes I–K) of near-saturating levels of mitochondrial transcription factor. Comparable promoter specificity is evident in the reconstituted system and, as is the case for LSP specificity, HSP activity depends on added transcription factor. Lanes D, H, and L represent identical transcription reactions programmed with 16 µg/ml linearized cloned DNA retaining only the LSP, controls included to ensure equal LSP-specific activity in HS pool and the reconstituted PC systems. At the highest concentration of the HSP-bearing template assayed (4 µg/ml, lane G), numerous additional, factor-dependent or factor-stimulated bands (compare lanes G and K) appear in the PC-reconstituted transcription system. The significance of these products is obscure, but they probably reflect specific initiation at sites within the vector, perhaps due to homologies to mtDNA promoters, since the most intense signals correspond to species longer than the excised mtDNA insert. Their absence in the reactions cata-

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2 R. P. Fisher and D. A. Clayton, unpublished observations.
3 D. D. Chang, personal communication.
human mitochondrial transcription

Fig. 8. Reconstitution of HSP-specific run-off transcription. A, equal amounts (1.2 poly(dA-dT) units) of mtRNA polymerase activity purified through the heparin-Sepharose stage (lanes A-D) or through the phosphocellulose stage (lanes E-L) were assayed for HSP-specific run-off transcription at 0.25 μg/ml (lanes A, E, and I), 1.0 μg/ml (lanes B, F, and J), or 4 μg/ml (lanes C, G, and K) of a template lacking the LSP (H5'-A-60) or at 16 μg/ml of a template lacking the HSP (L5'-A-70) (lanes D, H, and L). Expected run-off products from the HSP and LSP are ~191 nucleotides (double arrowhead) and ~416 nucleotides (closed arrowhead) long, respectively. In lanes E-H, promoter selectivity was reconstituted by the addition of 4 μl of PC-purified mitochondrial transcription factor; lanes I-L contain no added factor. Final glycerol and Triton X-100 concentrations were 16 and 0.32%, respectively. B, copurification of HSP- and LSP-specific transcription factor activity on phosphocellulose. PC fractions 15–20 (4 μl each) were assayed for run-off transcription initiating at the HSP (double arrowhead) in the absence (−) or presence (+) of 1.2 units of mtRNA polymerase (mt RNAP) (PC fraction 10), assayed alone in the leftmost lane. Enhancement of HSP-directed transcription is maximal in fraction 17, corresponding to the peak of LSP-specific enhancement seen in Fig. 3. Reactions were carried out as for A at 0.25 μg/ml template bearing only the HSP.

Analyzed by the HS pool may be a function of different proportions of factor to polymerase and/or factor to DNA in the two systems.

Copurification of HSP- and LSP-specific Transcription Factor Activity

In the experiment shown in Fig. 8B, consecutive PC fractions 15–20 were assayed in the absence (−) or presence (+) of mtRNA polymerase (assayed alone in the leftmost lane) for run-off transcription initiating at the HSP. Specific transcription is dependent on added polymerase and peaks in fraction 17, together with LSP-specific activity (see Fig. 3). This copurification is suggestive of a single factor conferring specificity for both the H- and L-strand promoters of mtDNA. A second peak of HSP enhancement is seen in fraction 20, in contrast to the situation for the LSP (Fig. 3), where only a single peak is apparent. This raises the possibility that a second, HSP-specific transcription factor is eluted from phosphocellulose at still higher ionic strength.

Copurification of HSP- and LSP-specific, Factor-dependent mtRNA Polymerase

We were also able to demonstrate that a single RNA polymerase is probably responsible for both H- and L-strand transcription. A phosphocellulose column profile of HSP-specific, factor-dependent mtRNA polymerase activity is shown in Fig. 9. PC fractions 7–12 were assayed for run-off transcription both in the absence (−) and presence (+) of mitochondrial
and binds sequences upstream of SV40 genes transcribed by RNA polymerase II (19).

There is indirect evidence which leads us to anticipate a greater complexity. Comparisons of a crude S-130, heparin-Sepharose pool, and phosphocellulose-fractionated reconstituted transcription systems were made using both poly(dA-dT) and a linearized, cloned D-loop template (see “Results”). While the two more extensively purified systems (HS and PC) show nearly identical specific-to-nonspecific activity ratios, i.e., equal selectivity, the crude extract (S-130) displays a 100-fold higher selectivity for the LSP. We believe that these data are best interpreted as indicating the removal from the extract, by heparin-Sepharose chromatography, of additional factors capable of stimulating promoter-directed transcription selectively.

In the promoter mapping of Chang and Clayton (6) and Hixon and Clayton (8), a regulatory role was inferred for sequences upstream of the minimal promoters; their deletion or alteration decreased the efficiency, but not the accuracy, of initiation. It remains to be seen whether the transcription factor we have isolated acts at these upstream sequences or on the minimal promoter itself. It seems likely, though, based on quantitative comparisons of promoter-specific transcription at the three stages of purification, that the protein requirements elucidated here are indeed minimal ones.

Copurification of HSP- and LSP-specific mtRNA polymerase activities provides the most compelling evidence thus far that, at least in vitro, a single mtRNA polymerase is sufficient for both H- and L-strand transcription. Consistent with this notion are the similar responses of HSP- and LSP-specific run-off transcription, both in the HS pool and in similar preparations to variations of ionic strength (data not shown) and of temperature (Ref. 20; data not shown). These systems, however, contain both mtRNA polymerase and mitochondrial transcription factor, and possibly other activities which could influence these responses. At least two components of the transcription machinery are engaged by both promoters; for a given property to be properly ascribed to, say, RNA polymerase rather than transcription factors, would require that the complex series of reactions leading to a run-off product be broken down into individual steps (e.g., promoter recognition, initiation, elongation). We have taken an alternative approach, using run-off transcription assays, to monitor the separation of the system into individual components. Although we cannot rule out completely the existence of exactly copurifying transcription factors and/or RNA polymerases with distinct specificities for the HSP and the LSP, the data presented here support the conclusion that at least these two basic elements of the transcriptional machinery are common to both promoters.

Considerable variability in HSP-specific activity among different HS pool preparations has been noted (5–7, 20), whereas LSP-specific activity appears to be more constant; the heparin-Sepharose pool comprising the input of the present phosphocellulose column is relatively deficient in HSP-directed run-off transcription. This variability implies that additional factors will prove necessary for efficient H-strand transcription. The two promoters, while similar in sequence, are not identical and exhibit markedly different sensitivity to base substitution (8), further strengthening the case for different protein requirements. It is worth noting, however, that the cleared lysate shows a promoter preference (i.e., LSP over HSP) comparable to that seen after further purification. This observation argues that additional HSP-specific factors, if they exist, may be already depleted in this crude extract. Depletion or deficiency of a putative HSP-specific factor (or factors) not required for initiation at the LSP could explain

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**Fig. 9. Phosphocellulose chromatography of mitochondrial transcription factor-dependent, HSP-specific mtRNA polymerase activity.** Phosphocellulose fractions 7–12 (2 μl each) were assayed for run-off transcription initiating at the HSP in the absence (−) or presence (+) of 4 μl of transcription factor (mt TF), assayed alone in leftmost lane. The template was BamHI/EcoRI-digested plasmid H5'Δ-60, at a final concentration of 0.25 μg/ml. All reactions contained 2 μl of DEAE-Sepacel flow-through fraction (see explanation in text).
the different responses to increasing DNA concentration seen in run-off assays with LSP- and HSP-bearing templates. H-strand transcription plateaus at DNA concentrations below 1 μg/ml (Fig. 8A), whereas LSP-directed transcription increases approximately linearly over a wide range of DNA concentrations (data not shown). Alternatively, or additionally, mtDNA topology may influence HSP activity preferentially. Wu and Dawid (13) noted changes in strand selectivity of purified Xenopus laevis mtRNA polymerase upon denaturation of the mtDNA template. In general, the run-off assay using linearized templates seems to accentuate the LSP-HSP disparity in the unfractionated HS pool; more congruent levels are seen in assays employing supercoiled templates (6). Unfortunately, a direct test of the effect of supercoiling on transcription must await further purification steps to remove a contaminating topoisomerase activity (data not shown).

That the same combination of proteins can support aggressive transcription of the L-strand and only weak activity on the H-strand of linear mtDNA may have important implications for the regulatory function of the D-loop. Multiple control elements relevant both to transcription of the L-strand and to replication of the H-strand are positioned downstream of the LSP (21). A mechanism has been inferred from in vivo mapping studies where initiation by RNA polymerase at the LSP can give rise either to transcription of the L-strand in its entirety or to processed molecules priming H-strand DNA synthesis, which in turn can lead either to precisely truncated D-loop DNA strands or to a full round of DNA replication (22). Such numerous and complex pathways do not seem to exist for transcripts initiated at the HSP, the primary or sole function of which appears to be the production of rRNAs, tRNAs, and mRNAs for mitochondrial translation. Thus, maintenance of a steady state transcriptional apparatus. Mitochondrial RNA polymerases from in vivo mitochondria 

is similar. Such lack of conservation among these control elements implies a stringent species specificity in the transcriptional apparatus. Mitochondrial RNA polymerases from human cells (5), Neurospora (24), Saccharomyces (14), and Xenopus (13) have been characterized and display striking similarities with respect to size, ionic strength optima, divalent cation requirements, and template preferences. The existence of dissociable transcription specificity factors could resolve the seeming paradox of highly conserved RNA polymerases recognizing profoundly divergent promoter sequences.

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