Replication Protein A Is a Component of a Complex That Binds the Human Metallothionein IIA Gene Transcription Start Site*

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Previous studies revealed that sequences surrounding the initiation sites in many mammalian and viral gene promoters, called initiator (Inr) elements, may be essential for promoter strength and for determining the actual transcription start sites. DNA sequences in the vicinity of the human metallothionein IIA (hMTIIA) gene transcription start site share homology with some of the previously identified Inr elements. However, in the present study we have found by in vitro transcription assays that the hMTIIA promoter does not contain a typical Inr. Electrophoretic mobility shift assays identified several DNA-protein complexes at the hMTIIA gene transcription start site. A partially purified protein fraction containing replication protein A (RPA) binds to the hMTIIA gene transcription start site and represses transcription from the hMTIIA promoter in vitro. In addition, overexpression of the human 70-kDa RPA-1 protein represses transcription of a reporter gene controlled by the hMTIIA promoter in vivo. These findings suggest that hMTIIA transcription initiation is controlled by a mechanism different from most mammalian and viral promoters and that the previously identified RPA may also be involved in transcription regulation.

Analysis of a large number of mammalian gene promoters revealed the existence of a conserved 5′-TATA-3′ sequence centered at around -30 (relative to the start of initiation) and a conserved 5′-CA-3′ sequence with A at +1 (1). The TATA element binds TFIIID and directs RNA polymerase and other required basal initiation factors to assemble a stable preinitiation complex. The importance of a TATA element was demonstrated in studies where deletion of this element from a promoter resulted in spurious initiation and a low level of transcription (reviewed in Refs. 2 and 3). However, not all eukaryotic genes contain a TATA consensus sequence upstream of their transcription initiation. Promoter studies of some of these TATA-less genes suggest that transcription initiation relies heavily on sequences surrounding the start site called initiator (Inr) elements (4-7). In one study, a 17-base pair element surrounding the transcription start site of the terminal deoxynucleotidyltransferase gene was sufficient for accurate basal transcription of this gene both in vitro and in vivo. In the presence of either a TATA box or the SV40 21-base pair repeats, a greatly increased level of transcription initiates specifically from this element (7).

Inr elements can be divided into many categories. Although they were first discovered in TATA-less promoters, many TATA-containing promoters also possess Inr elements (7-12). Some Inrs are weakly bound by a general transcription factor such as TFII-I, RNA polymerase II, or TFIIID, but they appear to lack recognition sites for specific DNA binding proteins (9, 11, 13), whereas others contain high affinity recognition sites for specific DNA-binding proteins (6, 12, 14-16). There are many definitions for an Inr in the current literature. The original definition for an Inr was a discrete promoter element that can act alone or in concert with either a TATA box or upstream element to direct specific transcription initiation (7). Later, the term Inr was restricted to the terminal deoxynucleotidyltransferase Inr and elements that have sequence homology to the terminal deoxynucleotidyltransferase Inr (10). At another extreme, the Inr has been used to describe any promoter element that determines the transcription start site (17). More recently, Inr elements have been defined as elements that can localize a transcription start site and mediate the action of at least some upstream activators in the absence of a TATA box (18). Whatever the definition, Inr elements are widespread in mammalian and viral promoters. In one study, O'Shea-Greenfield and Smale (10) inserted start site regions from eight different promoters downstream of the SV40 21-base pair repeats and found that all except the HIV-1 promoter exhibited Inr activity. Subsequently, it was shown that the HIV-1 core promoter lacks a simple Inr element but contains a bipartite activator at the transcription start site (19). The important conclusion is that start site region from many but not all mammalian and viral genes contain Inr activity. In fact, before the discovery of Inr elements, it was found that many DNA elements surrounding the start site of transcription can activate transcription (for examples see Refs. 20-29). Given the apparent importance of Inr elements and the wide distribution of them in many mammalian and viral gene promoters, there is a definite need to understand the mechanism by which they function and how they may regulate transcription.

We have previously shown that the adeno-associated virus P5 promoter initiation region binds the transcription factor YY1 (12, 30). We have also found that this initiation sequence shares homology with the terminal deoxynucleotidyltransferase Inr and is important for accurate basal transcription. Further, partially purified YY1 can restore basal level transcription from a P5 Inr in a HeLa extract depleted of YY1 or a...
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Drosophila embryo extract devoid of YY1 activity, whereas a YY1-specific antibody can block the reactivation. This was one of the first studies to identify a cellular factor that can mediate transcription through an Inr element and place the P5 Inr in the distinct class of Inrs that bind a sequence-specific protein. Interestingly, a computer alignment of eukaryotic promoter initiation sequences (at the +1 nucleotide) revealed that the sequence immediately surrounding the human metallothionein II A (hMTIIA) gene transcription start site exhibits homology to the P5 Inr (P5, 5'-GGTCCATTGTTT-3'; hMTIIA, GCACCT-CACCAAG). This hMTIIA transcription start site is identical to a sequence found in a fish Xiphophorus maculatus promoter (31) and the promoter of the human α-2 macroglobulin gene (32). It also shares over 80% identity with a sequence located in the human androgen receptor promoter (33). Although extensive analysis of the 5'-flanking regions of this promoter revealed a variety of trans-acting factors that bind to different upstream cis-acting sites (34-38), the role of the initiation region in basal transcription regulation is not known. A detailed study of the importance of the hMTIIA initiation site and characterization of possible factors that may interact with this region has not yet been done.

In spite of the homology to known Inrs in other mammalian and viral gene transcription start sites, we demonstrate in these studies that the hMTIIA transcription start site lacks a functional Inr element. More interestingly, we have demonstrated that several proteins bind to this transcription start site and repress transcription. One of these proteins has been identified as the human replication protein A (RPA), originally found as a multisubunit protein being absolutely required for SV40 DNA replication (39-41). This observation suggests that in addition to its role in DNA replication, genetic recombination (42, 43), and DNA repair (44-46), RPA may function as a DNA sequence-specific binding transcription factor.

MATERIALS AND METHODS
Plasmids—pMLTATA/P5+1, which contains a TATA box from the adenovirus major late promoter and −7 to +11 (simplified as +1) sequence from the adenovirus-associated virus P5 promoter, has been described (12). pMTIIA+1, pMLTATA/hMTIIA+1, and pSpI/hMTIIA+1 were constructed as described for TdT and P5 plasmids used in similar studies (7, 12, 47) but with the oligodeoxynucleotides 5'-GATCCGAGGCGTGGTGGATTTTG-3'.

Purification of hMTIIA Binding Proteins—A nuclear extract was prepared from 100 liters of HeLa cells by the method of Dignam et al. (57). This extract (185 ml, 2.3 g) was dialyzed against buffer B (20 mMEDP, pH 7.9, 10% glycerol, 50 mM MgCl₂, 60 mM KCl, 1 mM DTT, 50 µg/ml bovine serum albumin, 0.5 mM EDTA, 0.05% Nonidet P-40, 0.1 or 1 µg/ml of poly(dI-dC)- and approximately 7 µg of HeLa cell extract or approximately 0.4 µg of purified protein in a 12-µl total volume of labeled specified buffer, and 0.5 units of avian myeloblastosis virus reverse transcriptase) were added to each sample and incubated for 1 h at 42°C. 32P-Labeled extension products were extracted with phenol-chloroform, precipitated with ethanol, suspended in formamide loading dye, and separated on 8% sequencing gels.

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Electrophoretic Mobility Shift Assays—Single-stranded oligodeoxynucleotides were labeled individually with [γ-32P]ATP and T4 polynucleotide kinase at 16°C, heated to 65°C, and allowed to slow cool to room temperature. Each reaction contained 20 fmol of labeled DNA, 12 mM HEPEs, pH 7.9, 10% glycerol, 5 mM MgCl₂, 60 mM KCl, 1 mM DTT, 50 µg/ml bovine serum albumin, 0.5 mM EDTA, 0.05% Nonidet P-40, 0.1 or 1 µg/ml of poly(dI-dC)- and approximately 7 µg of HeLa cell extract or approximately 0.4 µg of purified protein in a 12-µl total volume of labeled specified buffer, and 0.5 units of avian myeloblastosis virus reverse transcriptase) were added to each sample and incubated for 1 h at 42°C. 32P-Labeled extension products were extracted with phenol-chloroform, precipitated with ethanol, suspended in formamide loading dye, and separated on 8% sequencing gels.

Purification of hMTIIA+1 Binding Proteins—A nuclear extract was prepared from 100 liters of HeLa cells by the method of Dignam et al. (57). This extract (185 ml, 2.3 g) was dialyzed against buffer B (20 mMEDP, pH 7.9, 10% glycerol, 50 mM KCl, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT) and then applied to a 2.6 x 40 cm P11 phosphocellulose column. After washing with buffer A, bound proteins were eluted with a 1.5-liter gradient of buffer C (50 mMEDP, pH 7.9, 10% glycerol, 50 mM KCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 0.5 mM DTT) and then applied to a 1.6 x 20 cm DEAE-Sephaloc column. Bound proteins were eluted with a 400-ml gradient from 0.05 to 1M KCl in buffer D. Protein-DNA complexes were detected by EMSA described above. Proteins forming complexes I and II eluted at about 250 mM KCl. These fractions were pooled (100 ml, 190 mg), dialyzed against buffer B (50 mM Tris-HCl, pH 7.5, 10% glycerol, 50 mM KCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 0.5 mM DTT), and then applied to a 1.6 x 20 cm DEAE-Sephaloc column. Bound proteins were eluted with a 400-ml gradient from 0.05 to 1M KCl in buffer E. Proteins forming complex I (34 ml, 21 mg) eluted at 250 mM KCl were partially resolved from those forming complex II. After dialysis against buffer C, the sample was applied to an FPLC HR5/5 Mono Q column, and bound proteins were eluted with a 20 ml gradient from 0.05 to 0.75M KCl in buffer F. Proteins forming complex III (8 ml, 14 mg) eluted at 350 mM KCl were resolved from those forming complex III.
The expected 79-nucleotide extension product is indicated by an arrow.

I. This fraction was then separated by gel filtration through a 1.6 × 60-cm Superdex 200 column (two identical fractions of 4-ml samples) that had been pre-equilibrated with buffer B containing 0.25 M KCl. Active fractions (20 ml, 2 mg) were dialyzed against Buffer C (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mM DTT, and 1 mM ammonium sulfate) and then applied to an FPLC HR 5/5 phenyl superose column. Bound proteins were eluted with a 20-mL linear gradient from 1 to 0 M ammonium sulfate in buffer C. Active fractions (10 ml, 200 μg) that eluted at about 0.3 M ammonium sulfate were dialyzed against buffer B and then fractionated by FPLC Mono Q chromatography as described above.

Proteolytic Digestion, HPLC Separation, and Microsequencing of hMTIIA-1 Binding Proteins—The most highly purified fractions that formed complex III were pooled and concentrated by ultrafiltration using a Centricon-10 filter (Amicon) as described by the manufacturer. Polyptides were separated by electrophoresis through a 10% denaturing polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane. Polypeptides on the membranes were visualized by staining with 0.1% Ponceau S in 5% acetic acid. After destaining of positive and negative factors, and the net result is a lack of Inr activity. Any rigorous test of these models requires the identification of DNA sequence that conveys Inr activity and characterization of proteins that interact with the hMTIIA start site DNA.

In Vitro Transcription Activity from the hMTIIA +1 Sequence Can Be Restored by Two Base Changes or Addition of hMTIIA +1 DNA—Five plasmids were constructed to contain DNA sequences mutated to mimic the P5 Inr. These plasmids were derived from pMLTATA/hMTIIA +1 (Fig. 1, lane 3, and Fig. 2A, lane 8), which contains the adenovirus major late TATA box upstream of the hMTIIA +1. In vitro transcription assays were performed, and the results are displayed in Fig. 2A. We found that mutations of the hMTIIA start site at nucleotides +2 and +3 (from CC to TT) imparted Inr activity. This is consistent with previous findings that the sequence requirement for Inr activity resides primarily between −1 and +3 with CATT being a much stronger Inr than CACC (18). If the lack of hMTIIA Inr activity is a result of a repressor acting on the hMTIIA +1, then one would predict that only hMTIIA +1mt2 would have lost its ability to bind this putative repressor. In contrast, if the lack of hMTIIA Inr activity is a result of the absence of an activator, then one would have to predict that hMTIIA +1mt2 and no other mutants gained the ability to bind this putative activator.

We reasoned that if the lack of an Inr activity from the hMTIIA +1 is due to the binding of a repressor, then the addition of DNA containing the hMTIIA +1 sequences into the in vitro transcription mixture should titrate out the repressor binding activity and restore transcription. In contrast, if the lack of an Inr activity from the hMTIIA start site is due to the lack of binding of an Inr activator, then the addition of DNA containing the hMTIIA start site sequences into the in vitro transcription reactions should have no effect on transcription activity. As shown in Fig. 2B, oligodeoxynucleotide containing hMTIIA +1 increased transcription initiation from a pMLTATA/hMTIIA +1 template (compare lane 1 with lanes 2–4). This activation of transcription is highly specific and requires specific hMTIIA +1 sequences, and as a mutated sequence (which presumably can no longer bind the putative repressor) cannot compete with the repression activity (Fig. 2B, lane 5); sequences that cannot restore transcription activity (Fig. 2A, lane 7, which presumably retains binding to the putative repressor) effectively competed with the repression activity (Fig. 2B, lane 6). Furthermore, excess hMTIIA +1 oligodeoxynucleotides have no effect on transcription of the P5 Inr (Fig. 2B, compare lanes 7 and 8). Taken together, this competition experiment suggests that the hMTIIA +1 sequence binds...
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A

1. no template
2. pMLTATA/PS1
3. pMLTATA/hMTIIA+1mt1
4. pMLTATA/hMTIIA+1mt2
5. pMLTATA/hMTIIA+1mt3
6. pMLTATA/hMTIIA+1mt4
7. pMLTATA/hMTIIA+1mt5
8. pMLTATA/hMTIIA+1

1 2 3 4 5 6 7 8

B

template
pMLTATA/hMTIIA+1
pMLTATA/hMTIIA+1mt2
pMLTATA/hMTIIA+1mt5
pMLTATA/hMTIIA+1mt4
pMLTATA/hMTIIA+1mt3
pMLTATA/hMTIIA+1mt1

1 2 3 4 5 6 7 8

Fig. 2. In vitro transcription and competition experiments with hMTIIA+1 and substitution mutants. A, the expected cDNA product of 79 nucleotides is indicated by an arrow (lower panel). Double-base substitution mutants were generated in the pMLTATA/hMTIIA+1 plasmid and tested by in vitro transcription and primer extension analysis as described in the legend to Fig. 1 and under “Material and Methods.” Each mutant nucleotide is denoted by an asterisk. B, in vitro transcription was performed as in panel A but in the presence of 0.01 pmol (lane 2), 0.1 pmol (lane 3), 1 pmol (lanes 4–6 and 8), or 10 pmol (lanes 10 and 11) of the indicated oligodeoxynucleotides.

detect specific DNA-binding proteins by EMSA. As shown in Fig. 3, when analyzed with HeLa nuclear extract, five sequence-specific DNA-protein complexes were detected. The formations of these complexes were prevented by the addition of excess unlabeled hMTIIA+1 oligodeoxynucleotide (Fig. 3, lanes 3–5) but not by an unrelated oligodeoxynucleotide containing the binding site for the transcription factor AP1 (Fig. 3, lanes 9–11). Interestingly, an oligodeoxynucleotide consisting of the P5 Inr sequence can also effectively inhibit formation of one of the complexes (complex III, Fig. 3, lanes 6–8). This complex probably does not contain YY1 protein, because complexes generated with binding sites corresponding to the P5 Inr did not migrate identically, and antisera directed against YY1 did not affect this complex (data not shown).

Replication Protein A Binds the hMTIIA+1 DNA Sequence—To identify the protein(s) responsible for the formation of complex III, the DNA binding activity was purified from a HeLa nuclear extract as described under “Materials and Methods.” After the final FPLC Mono Q column, fractions were assayed for DNA binding activity (Fig. 4A). The protein content of these fractions was also analyzed by Coomassie Blue staining after separation by denaturing gel electrophoresis (Fig. 4B). From these results it was apparent that the DNA binding activity had not been purified to homogeneity. However, two polypeptides with molecular masses of 70 and 32 kDa co-eluted with the DNA binding activity (Fig. 4). The peak fractions (16–19) were pooled and concentrated prior to separation by denaturing gel electrophoresis. After the transfer of polypeptides to a polyvinylidene difluoride membrane, the portion of the membrane that contained the 70-kDa polypeptide was excised and subjected to digestion with trypsin. The resultant peptides were separated by reverse phase HPLC, and the amino acid sequences of two peptides were determined. A search of the data bases with the peptides, IGNPVPYNEGLGQPQ and SGGVGSGNTNWK, revealed identical sequences within the 70-kDa subunit (RPA-1) of the trimeric human RPA (48).

To determine whether complex III indeed contains RPA, we performed supershift experiments. A binding reaction mixture containing 32P-labeled hMTIIA+1 DNA and a HeLa nuclear extract was incubated with a monoclonal antibody to the 70-kDa RPA-1, a monoclonal antibody to the 32 kDa RPA subunit,
HeLa cells also can be supershifted by an RPA antibody (produced by the partially purified DNA binding activity from lanes 6 and 8). As a negative control, a protein-DNA complex formed (lane 5) with factor Sp1. As shown in Fig. 5, the monoclonal antibody to an unrelated protein (transcription factor Sp1). As shown in Fig. 5A, complex III was removed (supershifted and masked by complex I) when reacted with an antibody to RPA (lanes 3 and 4) but not an antibody to Sp1 (lane 5). As a negative control, a protein-DNA complex formed by YY1 was not affected by the RPA-1 antibody (compare lane 6 with lanes 7 and 8). Furthermore, protein-DNA complex produced by the partially purified DNA binding activity from HeLa cells also can be supershifted by an RPA antibody (lanes 11 and 12). Taken together, these data provide strong evidence that one of the hMTIIA+1 binding activities (complex III) contains RPA.

To further confirm that RPA binds the hMTIIA+1 sequence, we repeated the EMSA using a separate source of RPA proteins obtained from HeLa cells (kindly provided by J erard Hurwit, Sloan-Kettering Cancer Center). This protein was purified to at least 95% homogeneity judged by SDS/polyacrylamide gel electrophoresis and silver staining. As expected, purified RPA binds the hMTIIA+1 sequence (Fig. 5B, compare lanes 1 and 2); and based on competition assays, the binding is highly specific (lanes 3 and 4).

**RPA Can Repress Transcription**—To determine the effect of RPA binding to the hMTIIA+1 DNA, we performed in vitro transcription experiments with nuclear extracts derived from HeLa cells in the presence and the absence of partially purified hMTIIA+1 binding proteins (partially purified RPA). When 1.2 or 2.4 μg of partially purified RPA was added to a reaction mixture containing the hMTIIA promoter with sequences from −286 to +73, the level of correctly initiated transcripts decreased (maximal repression was approximately 20 fold; Fig. 6A, compare lane 3 with lanes 4 and 5).

To be certain that RPA was not a contaminant in the partially purified protein fractions and that the repression effect was not due to another protein, we cotransfected a plasmid expressing the 70-kDa RPA-1 and an hMTIIA promoter reporter plasmid into CV1 cells and assayed for CAT activities. As shown in Fig. 6B, cotransfection with 4·5CAT plus pCMV-RPA(+) but not pCMV-RPA(−) caused a modest decrease in CAT expression. This low level of repression may be due to the fact that there could be other factors, such as other subunits of RPA or other hMTIIA+1 binding proteins, which are required in combination with the transfected RPA-1 in order to produce maximum repression. Also, the intracellular concentration of RPA-1 may be sufficiently high to negate the full repression effect of an additional source of protein supplied by transfection. Nevertheless, these data argue that RPA-1 binds the hMTIIA+1 sequence and represses transcription.

We have shown that overexpression of RPA-1 does not reduce activities from the Rous sarcoma virus, the simian virus 40, and the human immunodeficiency virus promoters. Taken together, data suggest that overexpression of RPA-1 can repress transcription from the hMTIIA promoter and is not generally cytotoxic.

To address whether the observed repression by RPA-1 is due to its ability to bind near a promoter, we constructed RPA-1 chimeric proteins with an added DNA binding specificity. When Gal4-RPA1(−) was cotransfected with the target plasmid pGal4TKCAT, a marked repression of CAT activity was observed (Fig. 6C, compare lanes 1 and 2). Neither a Gal4 DNA-binding domain alone or a nonfusion RPA-1 protein repressed transcription (compare lanes 1 and 3 and lanes 4 and 5). Also, repression was dependent on the presence of the Gal4-binding sites because CAT expression was not affected when TKCAT, lacking Gal4-binding sites, was used as a target (compare lanes 6 and 7).

Partially Purified RPA Fractions Bind Double-stranded but Not Single-stranded hMTIIA+1 DNA—Human RPA has high affinity for single-stranded DNA and low affinity for RNA and double-stranded DNA (39–41, 63–65). When binding to single-stranded DNA, RPA interactions are partially sequence dependent with an RPA monomer occupying about 30 nucleotides. To address the possibility that RPA was a contaminant in...
Fig. 6. Transcriptional effects of RPA. A, transcription was performed as described in the legend to Fig. 1 and under "Materials and Methods" with the following modifications: in vitro transcription was performed by preincubating template p4′5CAT at 0°C with either buffer alone (lane 3) or 1.2 μg (lane 4) or 2.4 μg (lane 5) of partially purified hMTIIA+1 binding protein for 15 min before the addition of HeLa nuclear extract. Transcription was initiated by the addition of ribonucleoside triphosphates and was allowed to proceed for 1 h at 30°C. RNA was analyzed by primer extension with a CAT primer (5′-GCCATTGG-GATATATCAACGGTGG). Lane 1 contains molecular weight markers derived from 32P-labeled, HinfI-digested, φX174 DNA. The expected DNA products of 170 and 173 nucleotides are indicated by an arrow. B, assay results with hMTIIA promoter CAT construct (5 μg) transfected into CV1 cells in the presence or the absence of a plasmid encoding the 70-kDa RPA-1 protein. The ratio of chloramphenicol acetylation in cotransfections with the RPA plasmids (5 μg each) to acetylation in cotransfection with the control pCMV plasmid (5 μg) is shown as relative CAT activity, after correction for transfection efficiency using an internal control of β-galactosidase activity. The numbers are the mean ± S.D. from two separate experiments. C, representative assay results of CV1 cells cotransfected with 10 μg each of pGal4TKCAT (lanes 1-5) or pTKCAT (lanes 6-8) and 20 μg each of pG4 (lanes 1 and 6), pG4-RPA(+) (lanes 2 and 7), pG4-RPA(−) (lanes 3 and 8), pCMV (lane 4), or pCMV-RPA(+) (lane 5), as indicated.

### Table I: Effects of RPA-1 on unrelated promoters

| Reporter/effector | pCMV (pCMV/Amp) | pCMV-RPA(+) |
|-------------------|-----------------|-------------|
| pRSVCAT           | 85.3 ± 0.6      | 94.5 ± 1.7  |
| pSV2CAT           | 45.6 ± 14.9     | 61.5 ± 9.3  |
| pHIV1CAT          | 16.4 ± 0.2      | 20.4 ± 9.4  |

We originally set out to test the hypothesis that the hMTIIA+1 sequence functions as a typical transcription Inr and to identify cellular factors that activate transcription of the hMTIIA promoter by binding to the site of initiation. Instead, we found that the hMTIIA+1 sequence binds proteins that mediate transcriptional repression. Five specific protein-DNA interactions were identified, suggesting a novel role for RPA in transcriptional regulation.
complexes are formed when HeLa nuclear extract is incubated with the hMTIIA+1 sequence. In this report, we describe the partial purification of the proteins responsible for one of these complexes, complex III. Polypeptides of 70 and 32 kDa co-elute with complex III formation. Amino acid sequencing of peptides from the 70-kDa protein identified this polypeptide as the 70-kDa subunit of replication protein A (41) (also known as RPA (39) or SSB (40, 65)). RPA is a trimeric DNA-binding protein with molecular weights of 70, 32, and 13 kDa that is evolutionarily conserved from yeast to human (67, 68). Based on the amino acid sequence identity and the molecular masses of the polypeptides that co-elute with complex III formation, it appears that at least two subunits of replication factor A are present in the complex that binds specifically to the hMTIIA+1 double-stranded DNA sequence.

RPA was initially identified as an essential factor for the in vitro replication of plasmid molecules containing the SV40 origin (39–41). Subsequently, it has been shown that RPA is a single-stranded DNA-binding protein, which interacts with DNA helicases such as SV40 T antigen and DNA polymerases (69). The observation that the Saccharomyces cerevisiae genes encoding the three RPA subunits are all essential is consistent with the predicted requirement for this complex in chromosomal replication (42, 68). More recently, RPA has been shown to be required for the incision step of DNA nucleotide excision repair (46), to be involved in genetic recombination (42, 43), and to bind to transcriptional activators such as Gal4, VP16, and p53 (70–72).

Our finding that the multifunctional RPA also binds specifically to the transcription initiation site of the hMTIIA promoter and may be involved in transcriptional regulation is both exciting and intriguing. While our work was in progress, Singh and Samson (73) reported that S. cerevisiae RPA binds specifically to two URS elements in the 3-methyladenine DNA glycosylase gene. Because similar sequences that function either as URS or UAS elements have been identified in the promoters of at least 11 genes that are involved in DNA metabolism, in particular DNA repair, it suggests that RPA may regulate a group of genes that are involved in the cellular response to DNA damage. Interestingly, expression of the human metallothioneine gene is also induced following DNA damage or treatment with heavy metals, hormones, and other agents (74–83). Thus, a similar regulatory pathway involving RPA may exist in mammalian cells.

How might RPA complex bind to the hMTIIA+1 sequence and repress transcription? First, just as the SV40 T antigen binding to the SV40 early promoter inhibits binding of transcription initiation complex to DNA (84–86), RPA or proteins associated with RPA may bind to the hMTIIA+1 sequence and prevent the binding of an Inr-binding protein that activates transcription. Second, it is possible that RPA may block the activation of an Inr-binding protein. For example, there may be activators that bind the hMTIIA+1 sequence simultaneously with the RPA complex and are inactivated by formation of a heterodimer with one of the RPA subunits. Finally, RPA may be a genuine transcription repressor composed of repression domains. Currently, repressing domains that have been identified in other proteins are commonly rich in alanine, glutamine, and/or proline, but no clear amino acid sequence similarities exist between the different repressing domains (reviewed in Ref. 87–89). A causal inspection of the RPA protein sequences has failed to reveal any obvious resemblance to well-characterized transcriptional repression motifs or to repression domains present in other transcription factors.

In addition to the complex that contains RPA, we have identified four other specific complexes that bind the hMTIIA+1 sequence. Here, we show that the hMTIIA+1 sequence not only failed to activate transcription but actually is regulated by a cellular negative factor. This repression phenomenon at the transcription start site is not unprecedented; Wiley et al. (62) described an activity (IBPs) that binds to the transcriptional initiation site of the SV40 major late promoter and represses transcription. Whether this type of regulation is only limited to a small set of promoters or whether it is a more widespread phenomenon remains to be determined.

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FIG. 7. EMSA and competition experiment of binding to oligodeoxynucleotides containing hMTIIA+1. 32P-Labeled hMTIIA+1 double-stranded oligodeoxynucleotides were used as substrates for binding with HeLa nuclear extract. Single-stranded DNA competitor in lanes 2 and 3 is an oligodeoxynucleotide containing the (+) strand DNA of hMTIIA+1. Single-stranded DNA competitor in lanes 4 and 5 is an oligodeoxynucleotide containing the (−) strand DNA of hMTIIA+1. Double-stranded DNA competitor in lanes 6 and 7 is an oligodeoxynucleotide containing both strands of DNA of hMTIIA+1. The numbers above the lanes indicate the molar excess of unlabeled DNAs.
