Inhibition of in Vivo and in Vitro Transcription by Monoclonal Antibodies Prepared against Wheat Germ RNA Polymerase II That React with the Heptapeptide Repeat of Eukaryotic RNA Polymerase II*  

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Wheat germ RNA polymerase II was used to raise monoclonal antibodies (mAbs) that cross-react with the largest subunit of calf thymus RNA polymerase II. Most of these mAbs were of the IgM isotype and were shown to react with a synthetic peptide containing the consensus sequence for the C-terminal heptapeptide repeat that has been found on the largest subunit of RNA polymerase II from a variety of eukaryotic organisms. A representative mAb (3WG2) was tested for its effect on transcription in both in vitro and in vivo systems. Antibody 3WG2 did not affect the transcription (elongation) of wheat germ RNA polymerase II on denatured calf thymus DNA. When HeLa cell nuclear extracts were preincubated with the mAb, run-off transcription from a promoter that contains a TATA box (the adenovirus-2 major late promoter) and from a promoter that does not contain a TATA box (the murine dihydrofolate reductase gene promoter = dhfr) was inhibited. Transcription from these promoters was also inhibited by the synthetic peptide containing the consensus sequence when it was conjugated to bovine serum albumin. HeLa cell nuclear extract in which the endogenous RNA polymerase II had been inhibited by the specific mAb was used to examine the ability of added mammalian RNA polymerase II that lacks the C-terminal domain to accurately transcribe specific genes. When calf thymus RNA polymerase II that lacked the C-terminal domain was added back to the inhibited extract, a discrete transcript that was initiated correctly was obtained with the adenovirus-2 major late promoter; however, no discrete transcript was observed from the mouse dhfr gene promoter. When injected into Xenopus laevis oocytes, antibody 3WG2 inhibited transcription of the human histone H2b gene (contains a TATA box) and the human U1 small nuclear RNA gene (does not contain a TATA box), but did not inhibit transcription from RNA polymerase I or RNA polymerase III promoters. These results indicate that the C-terminal heptapeptide repeat plays a critical role in promoter-directed transcription, although enzyme that lacks this domain can initiate from some promoters in vitro.

Eukaryotic organisms contain three types of nuclear RNA polymerases designated polymerase I, II, and III (A, B, and C, respectively). The most distinctive feature of these polymerases is the type of RNA that each synthesizes. RNA polymerase I synthesizes the precursors to large ribosomal RNA species. RNA polymerase II synthesizes the precursor to messenger RNA and small nuclear RNA (snRNA) species, with the exception of U6 snRNA. RNA polymerase III synthesizes a variety of small RNAs including transfer RNAs, 5 S ribosomal RNA, U6 snRNA, and 7SL and 7SK RNAs. All nuclear RNA polymerases are complex enzymes consisting of up to 14 distinctive subunits that share a common subunit pattern (for reviews see Lewis and Burgess (1982) and Sentenac (1985)). The basic subunit structure is the presence of two large subunits (approximately 140–240 kDa) and a variety of smaller subunits (less than 100 kDa).

Multiple forms of RNA polymerase II that differ only in the apparent size of the largest subunit have been reported (Dahmus, 1983; Guilfoyle et al., 1984; Roeder, 1976). The different forms of the enzyme isolated from mammalian cells have been referred to as polymerases IIo, IIA, and IIB (Roeder, 1976; Schwartz and Roeder, 1975). Form IIO has a subunit of approximately 240 kDa as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE); it is a minor form in preparations of purified enzyme but is a major form in mammalian cells (Kim and Dahmus, 1986). Form IIB has a subunit of approximately 180 kDa in SDS-PAGE and is generally the predominant form in purified RNA polymerase II preparations; form IIB presumably arises from the proteolytic cleavage of form IIO or IIA (Dahmus, 1983; Guilfoyle et al., 1984). Form IIA contains an intermediate sized largest subunit (190–220 kDa) in SDS-PAGE; it probably represents the actual gene product although it is uncertain if any intermediate proteolysis or differential phosphorylation has occurred (Kim and Dahmus, 1986).

Recently, the genes encoding the largest subunits of RNA polymerase II from yeast (Allison et al., 1985), mouse (Corden et al., 1985), Drosophila (Allison et al., 1988; Biggs et al., 1985; Zehring et al., 1988), and hamster (Allison et al., 1988) have been cloned. Sequence analysis of these genes has established an unusual heptapeptide repeat at the C-terminal end of the molecule. This sequence is highly conserved in mammals and

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1 The abbreviations used are: snRNA, small nuclear RNA; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; Ad2 MLP, adenovirus 2 major late promoter; PBS, phosphate-buffered saline; NTPS, nucleoside triphosphates.
yeast but less well conserved in *Drosophila*. The consensus sequence for this repeat is: Pro-Thr-Ser-Pro-Ser-Tyr-Ser. The number of repeats varies from 26 in the yeast to 52 in the mouse. Data suggest that this domain is lost due to proteolytic cleavage during purification to yield form IIB (Allison et al., 1985; Cadena and Dahmus, 1987; Corden et al., 1985). In addition, it appears that this domain is the site of phosphorylation of the largest subunit in *vivo* (Cadena and Dahmus, 1987; Dahmus, 1981), and the form that can be cross-linked to nascent mRNA contains the phosphorylated domain (Cadena and Dahmus, 1987).

The precise role of this domain in the regulation of transcription complex formation has yet to be determined. Deletion analyses have established that approximately one-half of the total number of repeats for a given species are necessary for cell viability (Nonet et al., 1987; Allison et al., 1988; Bartolomei et al., 1988; Zehring et al., 1988). Christmann and Dahmus (1981) reported the isolation of a monoclonal antibody that reacted with the largest subunit but not with the proteolysed form of the molecule. This antibody inhibited run-off transcription from a variety of TATA box-containing clones but did not inhibit transcription (elongation) from denatured calf thymus DNA (Dahmus and Redinger, 1983). This observation has been interpreted to mean that the C-terminal domain plays a critical role in initiation of transcription. However, Zehring et al. (1988) recently reported accurate transcription with a reconstituted transcription system containing *Drosophila* transcription factors and *Drosophila* RNA polymerase II that lacks this C-terminal domain.

Using RNA polymerase II purified from wheat germ as an immunogen, we have been able to isolate several monoclonal antibodies (mAbs) that react with the largest, unproteolyzed subunit of RNA polymerase II from a variety of eukaryotic species. These mAbs were found to react with a synthetic peptide containing three repeats of the consensus sequence for the C-terminal domain. These mAbs were examined for their effect on transcription from a promoter that contains a TATA box and from a promoter that does not contain a TATA box in both *vivo* and *vito* systems. Using these mAbs, we were able to examine the effect of a mammalian antigen, we have been able to isolate several monoclonal antibodies (mAbs) that react with the largest, unproteolyzed subunit of RNA polymerase II that lacks this C-terminal domain.

**Isolation of mAbs—**Adult female Balb/c ByJ mice (Jackson Laboratories, Bar Harbor, ME) were injected intraperitoneally with three injections of 20 μg each of purified wheat germ RNA polymerase II administered 10 days apart. The first injection was emulsified with Freund's complete adjuvant (Difco), and the remaining injections were emulsified with Freund's incomplete adjuvant (Difco). The immune response was monitored by an enzyme-linked immunosorbent assay (ELISA), using normal mouse serum as a control. Mice with antibody titers greater than 1:1000 were injected intraperitoneally with 40 μg of unpurified RNA polymerase II in phosphate-buffered saline; this injection was administered at least 30 days after the last injection and 3 days before the fusion. Spleen cells were fused with either NS1 or SP2/0 myeloma cells, using 40% polyethylene glycol 1000 (J. T. Baker Chemical Co., Phillipsburg, NJ) according to standard methods (de St. Groth and Steenbekker, 1988). Fusions were screened for RNA polymerase II-reactive antibodies by ELISA. Hybridomas were cloned twice by limiting dilution. For the production of ascites fluid, hybridomas were injected into Balb/c ByJ mice that had been primed approximately 1 week before with Pristane (Sigma). The class of each antibody was determined by using an ELISA isotyping kit (Boehringer Mannheim). The control monoclonal IgM (28.13.35), that reacts with the mouse H2-k* antigen, was kindly provided by Dr. Amy Moser, Department of Oncology, University of Wisconsin-Madison and has been described (Ozato and Sachs, 1981). The control IgG1 (2F8) that reacts with the a subunit of *Escherichia coli* RNA polymerase has been described (Strickland et al., 1988).

**Purification of mAbs—IgM antibodies were precipitated from ascites fluid by the addition of 45% saturated (NH₄)₂SO₄. Precipitated material was dissolved in Tris-HCl buffer (0.05 M, pH 7.9 at 23°C) containing 0.15 M NaCl. After centrifugation, and the supernatant fluid was loaded onto a column (2.0 × 100 cm) of Sephacryl S-300 (Pharmacia). Column fractions were monitored by SDS-PAGE, the appropriate fractions from the void volume were pooled, and the IgM was concentrated by centrifugation in a Centricon 30 (Amicon, Danvers, MA). This preparation was purified by chromatography on protein A that was linked to Sepharose 4B (Pharmacia). Antibody concentration was determined by absorbance reading at 280 nm using an extinction coefficient (E₂₈₀) of 13.8. Purified mAbs were stored in aliquots at −70°C.

**ELISA Procedure—**Antigen contained in PBS was coated onto the wells of a polystyrene microtiter plate overnight at room temperature. The plates were blocked with 200 μl of 1% nonfat dry milk (Johnson et al., 1984) contained in PBS for 2 h at room temperature. After washing with PBS containing 0.1% Tween 20 (Sigma), 50 μl of antibody solution was added and allowed to react for 1.5 h at room temperature. After washing with PBS containing 0.1% Tween 20 (Sigma), 50 μl of an appropriate dilution of anti-mouse IgG (Hyclone, Logan, UT) or anti-mouse IgM (Boehringer Mannheim) that was prepared in goats and conjugated to horseradish peroxidase was reacted for 1.5 h at room temperature. After extensive washing, the reaction was developed with ortho-phenylenediamine (Sigma; 0.8 mg/ml in 0.5 M citrate buffer, pH 5.0). Reactions were quenched with 50 μl of 1 M H₃SO₄ and read at 490 nm on a Titertek microtiter plate reader (Flow Laboratories, Inc., McLean, VA).

Electrophoresis and Immunoblots—Proteins were separated by electrophoresis according to the method of Laemmli (1970) using either a 4–20% gradient gel (Integrated Separation Systems, Hyde Park, MA) or a homogeneous gel containing either 10 or 15% polyacrylamide and 0.1% sodium dodecyl sulfate (SDS-PAGE). Electrophoresis was performed on a Mighty Small gel apparatus (Hoeffer Scientific Instruments, San Francisco, CA). For immunoblots, proteins were transferred to nitrocellulose (Schleicher and Schuell) according to the method of Towbin et al. (1979), except that 0.05% SDS was used in the transfer buffer. Blots were blocked with either Tris-HCl buffer (0.01 M, pH 7.4 at 23°C) containing 0.15 M NaCl and 0.1% Tween 20 (TBST) or 1% nonfat dry milk. Blotted material was washed with antibody-containing culture medium or diluted ascites fluid (1:500 dilution in 0.01 M Tris, 0.15 M NaCl, 1% BSA) for 1.5 h at room temperature. After washing extensively with TBST, the antibody was detected with anti-mouse IgG that was prepared in goats and conjugated to alkaline phosphatase (Boehringer Mannheim) for 1.5 h at room temperature. After extensive washing with TBST, the reaction was detected by the addition of 0.075 M 5-bromo-4-chloro-3-indolyl phosphate (Sigma) and 0.02 M nitro blue tetrazolium (Sigma) contained in 0.01 M Tris, pH 9.5 at 23°C, 0.1 M NaCl, 0.05 M MgCl₂ (Blake et al., 1984).

Dot blots were performed according to the procedure for immunoblots except that proteins (60 μl of the appropriate concentration) were spotted onto nitrocellulose using a Bio-Dot microfiltration apparatus (Bio-Rad). Dot blots were blocked with TBST and reacted
with a 1:500 dilution of ascites fluid.

**Peptide and Peptide Conjugate—** A peptide with the sequence Cys-Pro-Thr-Ser-Pro-Ser-Tyr-Ser-Cys was synthesized at the University of Wisconsin Biotechnology Center using an Applied Biosystems automatic solid-phase peptide synthesizer. This peptide was separated from small molecules remaining from the synthesis by filtration on a column (1 x 15 cm) of Sephadex G-10 (Pharmacia). This peptide preparation was stored at -20 °C. The peptide was conjugated to BSA (Fraction V, Miles Scientific, Naperville, IL) by the glutaraldehyde method of Dignam and Baltimore (1982), using a peptide to BSA molar ratio of 30:1. A sample of BSA was also treated with glutaraldehyde to serve as a control. The peptide-BSA conjugate and glutaraldehyde-treated BSA were analyzed by SDS-PAGE. While both preparations appeared as smears on a Coomassie-stained gel, the peptide-BSA conjugate was of a distinctly higher molecular weight.

**Non-specific Transcription Reactions—** Transcription that is not dependent on promoter-directed initiation was measured by a method similar to that used by Daha and Kedinger (1983). Reactions volumes were 100 μl and contained diluted enzyme; 25 μg of heat-denatured calf thymus DNA (Worthington); 0.6 mM each of ATP, GTP, and CTP; 1 μCi of [γ-32P]ATP (ICN Radiochemicals, Irvine, CA) diluted to 1 μCi/0.1 nmol; 25 mM Tris-Cl (pH 7.9 at 23 °C); 8 mM MgCl2, and 25 mM (NH4)2SO4. Reactions were incubated at 30 °C for 10 min before they were stopped with 10 μl of 2.5% SDS and 0.15 mM sodium pyrophosphate. A portion (7 μl) of each reaction mixture was applied to diethylaminoethyl (DEAE) cellulose discs (McLeaster Research Equipment, Inc., Madison, WI). The filters were air-dried, washed five times with a solution of 5% (v/v) anhydrous dibasic sodium phosphate, washed once with double-distilled water, and washed once with ethanol. Finally, the filters were dried and scintillation fluid was added. For experiments that tested the effect of antibody, the enzyme was preincubated with purified mAb for 30 min at 30 °C before the nuclease triphosphates (NTPs) and templates were added.

**Templates—** Plasmid pBeEI has been described (Manley et al., 1980; Murphy et al., 1987) and contains the adenovirus 2 major late promoter (Ad2 MLP) inserted into the BamHI site of PR322. Digestion of this plasmid with HindIII (Promega, Madison, WI), yields a 1456-bp pair fragment containing the pBeE1 MLP. Promoter-directed transcription from this fragment yields a run-off transcript of 1496 bases initiating at the Ad2 MLP. Plasmid pE2825, containing the murine dihydrofolate reductase (dhfr) gene, was a kind gift of Dr. Peggy Farnham, McArldre Laboratory, University of Wisconsin-Madison and has been described (Farnham and Schimke, 1986). This plasmid was digested with PoulI (New England Biolabs), and the fragment was isolated by polycarramide gel electrophoresis and eluted from the gel. The accurately initiated run-off transcript from this fragment is 477 nucleotides (Farnham and Schimke, 1986). The plasmid containing the human U1 maxigene (formerly called B33) has been described (Murphy et al., 1987; Skuzeski et al., 1984). The construction of the Xenopus S maxigene has been described (Sakonju et al., 1988). Plasmid phH2b-WT contains the human histone H2b gene from Dr. C.J. Dahlgren, Department of Physiological Chemistry, University of Wisconsin-Madison; this gene was originally cloned by Hazel Sive and Robert Roeder, Rockefeller University, New York. The human histone H2b gene was subcloned into pGEM-ZF(+) (Promega) by standard methods.

**Promoter-directed Transcription Reactions—** Run-off transcription reactions were carried out in HeLa cell nuclear extracts prepared by the method of Dignam et al. (1983), except that several extracts were pooled, precipitated with 66% ammonium sulfate, resuspended in one-quarter the original volume, dialyzed against buffer D for 8 h, and frozen in aliquots at -70 °C. Final protein concentration was approximately 25 mg/ml.

**Transcriptions using the Ad2 MLP** were performed in reaction volumes of 25 μl and contained an appropriate amount of diluted nuclear extract; 1 μg of plasmid DNA digested with the appropriate restriction enzyme; 7.5 mM MgCl2, 0.4 mM ATP, CTP, and GTP; 0.02 mM GTP, and 10 μCi of [α-32P]GTP (Du Pont-New England Nuclear) using the Ad2 MLP. Promoter-directed run-off transcription using the Ad2 MLP was performed in a reaction volume of 25 μl and contained an appropriate amount of diluted nuclear extract, 250 ng of isolated DNA fragment, 6 mM MgCl2, and the NTP concentrations listed above. For both promoters the diluted extract, template, and MgCl2 were preincubated for 10 min at 37 °C before the transcription reaction was initiated by the addition of NTPs and incubated at 30 °C for 30 min for the Ad2 MLP and at 24 °C for 15 min for the dhfr promoter. All transcription reactions were terminated by the addition of 100 μl of a solution containing 0.5 μg of protease K/ml and 0.5% SDS; this reaction was incubated for 30 min at 37 °C before the samples were processed by phenol extraction, ethanol precipitation (Murphy et al., 1982). Electrophoresis was performed on 2× 30 × 0.06 cm gels containing 7.5% polyacrylamide (30:0.8) and 30% (w/v) urea. Gels were run at 45 watts constant power (1500–1650 V) for 3 h.

**In Vitro Transcription** was performed by standard methods (Ausubel et al., 1987). The primer, ranging from +46 to +64 with respect to the Ad2 MLP transcriptional start at +1, was prepared by the University of Wisconsin Biotechnology Center.

**Oocyte Injections—** Injections were performed by the method of Murphy et al. (1987) except that the oocytes were centrifuged at 750 × g for 10 min immediately prior to injections (Lund et al., 1987) and the appropriate amount of purified mAb was co-injected with 0.25 μCi of [α-32P]GTP, 1 ng of each supercoiled plasmid DNA carrying the human gene, and 0.25 ng of supercoiled plasmid containing the Xenopus S maxigene. Oocytes were processed individually, and one-third of the RNA from each was analyzed by electrophoresis (Murphy et al., 1987) to identify the oocytes that had been effectively injected as determined by the presence of the S S maxigene transcript. These samples were pooled to obtain composite oocyte data.

**RESULTS**

**Properties of Wheat Germ RNA Polymerase II as an Immunogen—** RNA polymerase II isolated from wheat germ contains a large proportion of the form containing the unproteolyzed largest subunit (Fig. 1). Spleen cells from mice immunized with purified wheat germ RNA polymerase II were fused with mouse plasmacytoma cells. Resulting hybridomas were screened by ELISA, and the hybridoma supernatant fluids were subjected to electrophoresis in a 4–20% polyacrylamide gel. Wheat germ RNA polymerase II (WG RNA POL II) was purified by modification of the method of Johnson and Burgess (1975) and consists of subunits that are 220 kDa (and the proteolyzed form that is 180 kDa), 140, 40, 27, 25, 24, 20, 17.8, 17, 16.5, 16, and 14.5 kDa. Calf thymus RNA polymerase II (CT RNA POL II) was purified by the method of Hodo and Blatti (1977) and consists of subunits that are 214 kDa (and the proteolyzed form, most predominant in this preparation, that is 189 kDa), 140, 34, 25, 20, 15, 14, 13, and 10 kDa. Polypeptides of approximately 50 and 40 kDa in the calf thymus RNA polymerase II preparation contain the one-third of the RNA from each was analyzed by electrophoresis (Murphy et al., 1987) to identify the oocytes that had been effectively injected as determined by the presence of the S S maxigene transcript. These samples were pooled to obtain composite oocyte data.

**Fig. 1. SDS–PAGE and immunoblots of RNA polymerase II isolated from wheat germ and calf thymus.** Denatured enzymes were subjected to electrophoresis in a 4–20% polyacrylamide gel. Wheat germ RNA polymerase II (WG RNA POL II) was purified by modification of the method of Johnson and Burgess (1975) and consists of subunits that are 220 kDa (and the proteolyzed form that is 180 kDa), 140, 40, 27, 25, 24, 20, 17.8, 17, 16.5, 16, and 14.5 kDa. Calf thymus RNA polymerase II (CT RNA POL II) was purified by the method of Hodo and Blatti (1977) and consists of subunits that are 214 kDa (and the proteolyzed form, most predominant in this preparation, that is 189 kDa), 140, 34, 25, 20, 15, 14, 13, and 10 kDa. Polypeptides of approximately 50 and 40 kDa in the calf thymus RNA polymerase II preparation contain the one-third of the RNA from each was analyzed by electrophoresis (Murphy et al., 1987) to identify the oocytes that had been effectively injected as determined by the presence of the S S maxigene transcript. These samples were pooled to obtain composite oocyte data.
from the ELISA-positive cultures were assayed by immuno-
blot analysis to determine the subunit specificity. Approx-
imately 40% of the antibodies reactive in ELISA did not react
with material blotted from SDS-polyacrylamide gels. In a
typical fusion, the predominant immunoblot-reactive anti-
body that was isolated reacted most strongly with the 220-
kDa subunit of wheat germ RNA polymerase II, although
some antibodies that reacted with some of the smaller sub-
units were also detected. However, the antibodies that reacted
most strongly with the 220-kDa subunit reacted poorly or not
at all with the 180-kDa subunit (Fig. 1) which is the proteo-
lized form of the largest subunit (Lewis and Burgess, 1982)
and, by analogy to other RNA polymerase II molecules, is
believed to be the form that lacks the C-terminal domain.
Many antibodies with this reaction pattern have been isolated
and all, with the exception of one, are of the IgM isotype.

Five of these antibodies (designated 3WG2, 1CWG1,
1CWG2, 1CWG3, and 8WG16) were examined for cross-
reactivity with mammalian RNA polymerase II by using pu-
rified calf thymus RNA polymerase II. The immunoblot in
Fig. 1 shows a typical reaction of one IgM antibody (3WG2)
with RNA polymerase II purified from wheat germ and calf
thymus. It should be noted that the antibody reacted strongly
with the unproteolyzed largest subunit of calf thymus RNA
polymerase II even though the preparation did not contain
a significant amount of this form of the enzyme.

Epitope Specificity of mAbs—Because these mAbs did not
react well with the proteolyzed form of the largest subunit
of calf thymus RNA polymerase II, we suspected that the epi-
topes for these antibodies are located in the C-terminal hept-
tapeptide repeat. To test the reactivity of these mAbs with
this sequence, a peptide containing three repeats of the con-
sensus sequence was synthesized. To facilitate conjugation
of the peptide to a carrier molecule or polymerization of the
subunits in future experiments, cysteine residues were placed
on each end of the peptide. Some of the peptide was conju-
gated to BSA as described under “Materials and Methods.”

The antibodies were tested for reactivity with the peptide
and the peptide-BSA conjugate in both ELISA (Table I) and
dot blot assays (Fig. 2). All of the antibodies, with the exception
of 1CWG3, reacted with the peptide in the ELISA assay;
however, this antibody reacted well with the peptide-BSA
conjugate in this assay. All of the antibodies reacted with
both the peptide and the peptide-BSA conjugate in the dot
blot assay. No significant reactivity with glutaraldehyde-
treated BSA could be detected in either assay. In addition, no
reactivity could be detected in the dot blot assay when the
control monoclonal IgM (28.13.3S) or the control monoclonal
IgG (2F8) was used.

Effect of the mAbs on Nonspecific in Vitro Transcription—
We suspected that the antibodies that we isolated were similar
in specificity to the one reported by Dahmus and Kedinger
(1983). Therefore, we tested the effect of these mAbs in
nonspecific and promoter-directed transcription assays.

A preparation of wheat germ RNA polymerase II that
contained approximately equal amounts of the unproteolyzed
and proteolyzed largest subunit as determined by SDS-PAGE
was selected. Antibody 3WG2 was conjugated to Sepharose
and used to deplete a wheat germ RNA polymerase II prepa-
ration of the form that contains the unproteolyzed largest
subunit. Analysis of the resulting material by SDS-PAGE
established that it contained only the form with the proteo-
lized largest subunit. When the depleted material was tested
for activity on denatured calf thymus DNA, approximately 2
µg incorporated 14,000 cpm of [3H]UTP. By comparison,
approximately 2 µg of material that was treated with control
Sepharose incorporated 33,000 cpm. Therefore, both forms of
the wheat germ enzyme are active in this assay.

This wheat germ RNA polymerase II preparation was treated
with increasing concentrations of either 3WG2 or control IgM
for 30 min at 30 °C before the transcription assays were initiated by the addition of denatured calf thymus
DNA and NTPs. Although there is considerable variability in
the values obtained from this assay, the results (Fig. 3)
indicate that elongation is not appreciably affected by the
binding of this antibody. The control IgM (28.13.3S) also
showed no appreciable effect in the nonspecific transcription
system.

Antibodies 1CWG1, 1CWG2, 1CWG3, and 8WG16 were all
tested in this assay. Like 3WG2, none of these mAbs showed

### Table I

Characterization of mAbs with respect to isotype and reactivity with the synthetic peptide and the peptide-BSA conjugate in ELISA reactions

<table>
<thead>
<tr>
<th>Antibody designation</th>
<th>Isotype</th>
<th>Optical density readings in ELISA*</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>WG RNA polymerase II</td>
</tr>
<tr>
<td>3WG2</td>
<td>IgM</td>
<td>1.48</td>
</tr>
<tr>
<td>1CWG1</td>
<td>IgM</td>
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<td>1CWG3</td>
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<td>1.25</td>
</tr>
<tr>
<td>8WG16</td>
<td>IgG2a</td>
<td>1.27</td>
</tr>
</tbody>
</table>

* ELISA reactions were performed with ascites fluid diluted 1:500.

** Wells coated with 50 µl of wheat germ (WG) RNA polymerase II (1.4 µg/ml).

*** Wells coated with 50 µl of synthetic peptide (20 µg/ml).

**** Wells coated with 50 µl of synthetic peptide conjugated to BSA (2.0 µg/ml; based upon the amount of BSA used in the conjugation procedure).

***** Wells coated with 50 µl of BSA that had been treated with glutaraldehyde (2.0 µg/ml; based upon the amount of BSA used in the conjugation procedure).
The Ad2 MLP promoter contains a TATA box and is accurately transcribed by our HeLa cell nuclear extracts. HeLa cell nuclear extract was treated with 0.1, 0.25, 0.5 and 1.0 μg of 3WG2 or with 1.0 μg of control IgM for 30 min at 30°C. The template and MgCl₂ were added and the samples were incubated for 15 min at 30°C to allow the transcription complex to form. Transcription reactions were initiated by the addition of NTPs. The autoradiogram prepared from the RNA isolated from these reactions is presented in Fig. 5A. Antibody 3WG2 inhibited transcription from the Ad2 MLP at 0.5 and 1.0 μg per reaction. The control IgM at 1.0 μg had no detectable effect on the amount of transcript produced.

The effect of antibody 3WG2 on transcription from the mouse dhfr gene promoter was also examined. This promoter does not contain a TATA box and is accurately transcribed by our HeLa cell nuclear extracts. Preliminary experiments indicated that precubation of the antibody with the extract at 37°C was detrimental to some factor (possibly transcription factor Sp1) necessary for transcription of this gene. Therefore, HeLa cell nuclear extracts were incubated with 0.1, 0.25, 0.5, and 1.0 μg of 3WG2 or with 1.0 μg of control IgM for 60 min at 4°C. The template and MgCl₂ were added, and the reaction was incubated for 15 min at 24°C to allow appreciable inhibition of transcription using purified wheat germ RNA polymerase II and denatured calf thymus DNA (data not shown).

Effect of the mAbs on Promoter-directed Transcription—Antibodies that cross-react with wheat germ and calf thymus RNA polymerase II are likely to cross-react with HeLa cell RNA polymerase II. Immunoblots prepared using HeLa cell extracts were reacted with antibody 3WG2. Although several bands in the extract reacted strongly with the antibody, one of these reactive bands corresponded to a protein of approximately 220 kDa as judged by the reaction of the wheat germ RNA polymerase II run in parallel (Fig. 4). Many of the lower molecular weight protein bands that react with the antibody might be breakdown products of the subunit or even free C-terminal domain. The effect of this antibody on promoter-directed transcription was tested in HeLa cell nuclear extracts, using a promoter that contains a TATA box and one that does not contain this motif.
the transcription complex to form. Transcription reactions were initiated by the addition of NTPs. The results (Fig. 5B) indicate that some inhibition was evident at 0.5 μg of antibody and that inhibition was complete at 1.0 μg of antibody. The control IgM at 1 μg per reaction had no detectable effect on the amount of transcript produced.

To test for the presence of RNase activity in the antibody preparations, transcription reactions were terminated by the addition of 1 μg of α-amanitin/ml. Then the Ad2 MLP reactions were postincubated with 1 μg of antibody 3WG2 at 30 °C for an additional 30 min. The reaction containing the dhfr gene was incubated at 24 °C for an additional 15 min. The second to last lanes of Fig. 5, A and B, contain the RNA isolated from these reactions. No decrease in the amount of transcript could be detected; therefore, the decrease of transcription seen with the 3WG2 antibody was not due to RNase activity in the antibody preparation.

Antibodies 1CWG1, 1CWG2, 1CWG3, and 8WG16 were all tested in this assay, and all of these mAbs show similar inhibition patterns to 3WG2 when tested using the Ad2 MLP (data not shown).

Inhibition of Transcription by the Peptide Conjugate—Because these mAbs react with the heptapeptide repeat (Fig. 2) and inhibit promoter-directed transcription (Fig. 5) it was of interest to determine if the peptide and/or peptide-BSA conjugate could also inhibit transcription. HeLa cell extracts were preincubated with either the peptide, the peptide-BSA conjugate or glutaraldehyde-treated BSA before the addition of template and NTPs. Preincubations were performed for 30 min at 30 °C for the MLP and for 1 h at 4 °C for the dhfr gene promoter. The peptide was not able to inhibit transcription consistently at a concentration of 40 μg with either promoter. However, peptide that had been conjugated to BSA was capable of inhibiting transcription from the Ad2 MLP (Fig. 6A) and the dhfr gene promoter (Fig. 6B). Inhibition was complete at 160 μg of peptide conjugate; these concentrations are based upon the amount of BSA present because it was not known how much peptide was actually conjugated to the BSA. Glutaraldehyde-treated BSA was not able to inhibit transcription at 160 μg (Fig. 6, A and B).

The peptide, the peptide-BSA conjugate, and the glutaraldehyde-treated BSA were also tested in the nonspecific transcription assay, using wheat germ RNA polymerase II and denatured calf thymus DNA. None of these preparations had appreciable effect on the amount of RNA produced in the nonspecific assay (data not shown).

Effect of Different Forms of Calf Thymus RNA Polymerase II on Restoration of Activity—The data of Zehring et al. (1988) indicated that in their Drosophila system the C-terminal domain is not essential to achieve accurate in vitro transcription from a Drosophila promoter that contains a TATA box. Using the ability of mAb 3WG2 to inhibit HeLa cell RNA polymerase II, we were able to test the effect of different forms of mammalian RNA polymerase II in our HeLa cell system. Weil et al. (1979) showed that calf thymus RNA polymerase II could substitute for HeLa cell RNA polymerase II in HeLa cell extracts. Nuclear extracts were treated with 0.5 μg of mAb 3WG2 for 1 h at 4 °C. The the mAb-inhibited extract was supplemented with RNA polymerase II that had been isolated from calf thymus. One preparation of calf thymus RNA polymerase II was isolated by the procedure of Hodo and Blatti (1977) and contained essentially the proteolyzed form (180 kDa) of the largest subunit. The other preparation was purified by an immunofluoriness chromatography procedure using mAb 1CWG3; this preparation contained essentially the unproteolyzed (220 kDa) form of the largest subunit. The composition of the largest subunit in these preparations is compared in Fig. 7A. The enzyme prepared by the method of Hodo and Blatti (1977) contained a small amount of the form containing the heptapeptide repeat as revealed by the immunoblot using mAb 3WG2; therefore, this preparation was pretreated with 200 μg of mAb 3WG2/ml for 30 min before it was added to the extract.

When the Ad2 MLP was used as a template, a discrete transcript could be observed with both forms of the enzyme (Fig. 7B). Primer extension experiments confirmed that both transcripts were correctly initiated (data not shown). Accurate transcription from the mouse dhfr gene promoter could be restored only by the enzyme containing the C-terminal domain; no transcript was observed from this promoter when the proteolyzed enzyme was used (Fig. 7C).

To determine if the calf thymus RNA polymerase II containing the C-terminal domain was simply relieving the endogenous HeLa cell RNA polymerase II of inhibition by the antibody, one sample of the immunofluoriness-purified calf thymus RNA polymerase II was inactivated by treatment with 2 M KSCN and dialyzed against storage buffer before it was added to the mAb-inhibited nuclear extract. The addition of this enzyme to the mAb-inhibited nuclear extract did not restore appreciable activity (Fig. 7B).

Effect of mAbs on Transcription in Oocytes—To test the effect of mAb 3WG2 on transcription in vivo we cocajected the mAb with plasmids containing human genes into stage six Xenopus laevis oocytes. Therefore, it was necessary to establish that RNA polymerase II from X. laevis reacted with antibody 3WG2. Germinal vesicles were prepared from stage six oocytes by the method of Birkenmeier et al. (1978). These vesicles were immediately placed in SDS-PAGE sample buffer
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and disrupted by pipetting. Samples were boiled for 2 min and then run on SDS-polyacrylamide gels. Immunoblots were prepared using both antibody 3WG2 and the control IgM. The results (Fig. 4) show that several species of protein react with antibody 3WG2. One of these species appears to be approximately 220 kDa as judged by the reaction of the wheat germ RNA polymerase II run in parallel.

We selected genes whose transcripts are stable and are of a discrete size so that the transcript can be detected directly on an autoradiogram if 32P-labeled GTP is injected with the template. The human U1 snRNA gene is transcribed accurately in X. laevis oocytes (Murphy et al., 1982; Murphy et al., 1987), and the transcript is stable; this gene does not contain a TATA box in the promoter region. To distinguish the transcript from endogenous Xenopus U1 snRNA, the human U1 maxigene (Murphy et al., 1987) was used. The human histone H2b gene was used as an example of a promoter that does contain a TATA box. Supercoiled plasmids containing these human genes were co-injected with increasing concentrations of antibody 3WG2 or control IgM. In all cases, an additional plasmid containing the Xenopus 5 S RNA maxigene was also injected which served as an internal control for successful nuclear injection. RNA isolated from these oocytes was separated on denaturing polyacrylamide gels and autoradiograms were prepared. The results show that transcription of the human U1 snRNA gene (Fig. 8A) and the human histone H2b gene (Fig. 2B) could be inhibited by 30 and 60 ng of antibody 3WG2 per oocyte nucleus, but not by 12.5 ng of antibody per nucleus. Control antibody injected at 30 and 60 ng per nucleus did not inhibit transcription of the human genes.

**DISCUSSION**

Central to understanding the process of transcription is an understanding of how RNA polymerase interacts with transcription factors and promoter elements to faithfully accomplish initiation. This is particularly true with regard to the eukaryotic enzyme RNA polymerase II, which must transcribe an enormous variety of "house-keeping" and specialization genes. Recent information regarding the primary structure of the largest subunit of RNA polymerase II has contributed greatly to our knowledge of this enzyme. The most intriguing aspect of this primary structure is the unusual heptapeptide repeat that comprises the C-terminal domain. Several possible functional roles have been proposed for this domain (Allison et al., 1985; Allison et al., 1988; Corden et al., 1985; Nonet et al., 1987). This domain might provide sites for (i) phosphorylation of the enzyme, (ii) binding of transcription factor to the enzyme, or (iii) association of the enzyme with chromatin or other nuclear structures. Conceivably, all of these roles could help to regulate transcription. To date, there is evidence only for phosphorylation of this domain (Cadena and Dahmus, 1987), although nothing is known about what role phosphorylation might play in regulation of initiation.

**Epitope Specificity of mAbs**—Relative to preparations of RNA polymerase II isolated from other sources, preparations of wheat germ RNA polymerase II purified by the method of Jendrisak and Burgess (1975) contains a high proportion of the form with the unproteolyzed largest subunit (220 kDa). The nomenclature described for mammalian RNA polymerase II has not been applied to the wheat germ enzyme. In addition, nothing is known about the phosphorylation state of the wheat enzyme as it is isolated from wheat germ. Therefore, it is not known if the form containing the unproteolyzed largest subunit is homologous to the IIO or IIA form identified for calf thymus and HeLa cell RNA polymerase II (Cadena and

When wheat germ RNA polymerase II was injected into mice for the production of hybridomas, a large number of these hybridomas produced antibodies that reacted very strongly with the 220-kDa subunit but did not react well with the proteolyzed subunit (180 kDa). The gene for the largest subunit of wheat RNA polymerase II has not been cloned and sequenced. However, because all of these antibodies cross-reacted with calf thymus RNA polymerase II and the heptapeptide repeat seems to be highly conserved, it seemed likely that our antibodies also reacted with the heptapeptide repeat. All of our antibodies showed reactivity toward a synthetic peptide containing three repeats of the heptapeptide in dot blot assays (Fig. 2). Reactivity was not as strong toward the peptide in ELISA assays unless the peptide was conjugated to BSA (Table I). It is possible that the free peptide does not bind well to the polystyrene plate.

We conclude from these data that the C-terminal domain of wheat germ RNA polymerase II conforms to the consensus sequence reported for yeast, mouse, hamster, and Drosophila RNA polymerase II and that the epitopes for the antibodies are contained within the heptapeptide repeat. Considering the highly repetitive nature of the heptapeptide in the C-terminal domain, it is not surprising that the repertoire of monoclonal antibodies that react with this region are largely of the IgM isotype. Highly repetitive immunogens often elicit only an IgM response (Basten and Howard, 1973). Curiously, we have not been able to raise an antibody response in mice to either the peptide or the peptide-BSA conjugate.

Effect of the mAbs on Nonspecific in Vitro Transcription—The mAbs were tested for inhibition of nonspecific transcription, using denatured calf thymus DNA as the template. However, an important point to consider is that the mAbs described here and the mAb reported by Dahmus and Kedinger (1983) react only with the form of the enzyme that contains the 220-kDa subunit which is generally a small proportion of the molecules in preparations of purified mammalian RNA polymerase II. Because considerable variability in the relative affinities of these antibodies, the relative amounts of RNA polymerase II present, or the relative activities of the antibody preparations. Antibodies 1CWG1, 1CWG2, 1CWG3, and 8WG16 all inhibited transcription from the Ad2 MLP at approximately the same concentration as antibody 3WG2 (data not shown).

It was of interest to examine the effect of antibody 3WG2 on a promoter that lacked a TATA box in the promoter region. Therefore, we examined the effect of this antibody on transcription from the mouse dhfr gene promoter. Inhibition of transcription from this promoter was complete with 1.0 μg of antibody (Fig. 5B).

Recently extracts prepared from wheat germ have been shown to transcribe a plant promoter accurately (Yamazaki and Imamoto, 1987). We have prepared wheat germ extracts according to this procedure. This extract does not transcribe from the Ad2 MLP (data not shown), but we are currently examining this extract with other promoters. The effect of our mAbs will be assessed in the wheat germ extract as soon as a suitable promoter is identified.

Inhibition of Transcription by Peptide Conjugate—Although we were unable to inhibit transcription consistently by the addition of our synthetic peptide to HeLa cell nuclear extracts, we were able to inhibit transcription consistently from both the Ad2 MLP and the dhfr gene promoter by peptide that was conjugated to BSA (Fig. 6). The amount of peptide-BSA conjugate that was needed to affect transcription seemed excessively high (160 μg in HeLa cell extracts). However, the amount of conjugate added to the extracts was based upon the amount of BSA that was used in the conjugation procedure; thus most of the addition was BSA. The fact that our peptide contained only three repeats of the heptapeptide might account for the fact that it needed to be conjugated to a carrier protein to achieve a localized concentration of multiple sites that might mimic the native domain. It is also likely that we achieved some polymerization of the heptapeptide units by reaction through the terminal cytisines during glutaraldehyde treatment.

Restoration of Activity by Calf Thymus RNA Polymerase II—Inhibition of promoter-directed transcription by mAbs that react with the heptapeptide repeat and by a synthetic peptide that contained this repeat seemed to indicate that initiation of transcription was dependent upon this domain. However, we were able to confirm some of the results reported by Zehring et al. (1988) regarding the ability of the proteolyzed enzyme to accomplish accurate transcription in vitro. The proteolyzed calf thymus enzyme was able to transcribe from the Ad2 MLP but not from the dhfr gene promoter (Fig. 7).

Clearly, there is a promoter specificity present that might or might not be based upon the presence of a TATA box in the promoter. It is possible that genes that lack a TATA box might be more dependent upon the interaction of the C-terminal domain with DNA-bound transcription factors to

run-off transcription assays, using two different promoters. The Ad2 MLP is a widely studied promoter containing a typical TATA box structure. It is transcribed faithfully in HeLa cell nuclear extracts. This promoter was also examined by Dahmus and Kedinger (1983), and transcription from this promoter was inhibited by their mAb. Our mAb was also able to inhibit transcription from this promoter (Fig. 5A). Inhibition was demonstrated with 0.5 μg/reaction (20 μg/ml), but not with 0.25 μg/reaction (10 μg/ml). In contrast, Dahmus and Kedinger (1983) reported that inhibition could not be detected with their antibody at less than 180 μg/ml. This large discrepancy between the amounts of antibody needed to inhibit transcription from this promoter might reflect differences in the relative affinities of these antibodies, the relative amounts of RNA polymerase II present, or the relative activities of the antibody preparations. Antibodies 1CWG1, 1CWG2, 1CWG3, and 8WG16 all inhibited transcription from the Ad2 MLP at approximately the same concentration as antibody 3WG2 (data not shown).

Finally, this experiment addresses another important point. Typically monoclonal antibodies are not effective precipitating reagents due to the monovalent nature of epitope in the antigen. However, given the repetitive nature of the epitope in this antigen, the possibility exists that a precipitation event could simply remove the enzyme from solution. Because no decrease in activity was seen in these assays, it can be assumed that active enzyme was not being removed from the solution.

Effect of the mAbs on Promoter-directed Transcription—Antibody 3WG2 reacts with what appears to be the largest subunit of RNA polymerase II in HeLa cell extracts (Fig. 4). This antibody was examined for its effect on transcription in HeLa cell nuclear extracts (Fig. 5A). Inhibition was demonstrated with 0.5 μg/reaction (20 μg/ml), but not with 0.25 μg/reaction (10 μg/ml). In contrast, Dahmus and Kedinger (1983) reported that inhibition could not be detected with their antibody at less than 180 μg/ml. This large discrepancy between the amounts of antibody needed to inhibit transcription from this promoter might reflect differences in the relative affinities of these antibodies, the relative amounts of RNA polymerase II present, or the relative activities of the antibody preparations. Antibodies 1CWG1, 1CWG2, 1CWG3, and 8WG16 all inhibited transcription from the Ad2 MLP at approximately the same concentration as antibody 3WG2 (data not shown).

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accomplish accurate initiation. Additional promoters need to be examined to determine if this difference is related to promoter structure. Perhaps the strong viral promoter is not as fastidious in its requirement for precise transcription complex formation which might require, or be enhanced by, the unique C-terminal domain. It is also possible that the addition of a large amount of purified RNA polymerase II might override subtle effects of the domain.

Effect of mAbs on Transcription in Oocytes—Finally, we have examined the effect of mAb 3WG2 on transcription of the human U1 snRNA and human histone H2b genes in X. laevis oocytes. The fact that antibody 3WG2 inhibits transcription of the U1 gene is consistent with the belief that U1 is transcribed by RNA polymerase II. To date, this belief has been based largely on sensitivity to α-amanitin, the presence of a methylated guanosine cap at the initiating nucleotide, and the general structure of the promoter (Murphy et al., 1982; Skuzeski et al., 1984; Murphy et al., 1987; Gunderson et al., 1988). The U1 snRNA gene lacks a TATA box in the promoter region. Antibody 3WG2 also inhibits the transcription of the human H2b histone gene; this promoter contains a TATA box.

Antibody 3WG2 did not inhibit transcription of the endogenous Xenopus 5.8 S RNA, an RNA polymerase I transcript. A plasmid containing the 5 S maxigene was co-injected with the U1 template and the antibody preparations. This was included primarily to assess successful nuclear injections. However, antibody 3WG2 had no effect on the transcription of the 5 S maxigene or the endogenous 5 S gene (Fig. 8A), both RNA polymerase III transcripts.

The experiments in Fig. 8 show that between 12.5 and 30 ng of 3WG2 are necessary to inhibit transcription. Like the immunoblots prepared from HeLa cell extracts, immunoblots prepared from germinal vesicles (Fig. 4) show several species of proteins that react with antibody 3WG2. We believe that some of these bands correspond to free C-terminal domain, but the identity of these proteins is unknown. Therefore, we cannot eliminate the possibility that the interaction of the antibody with one of these proteins is responsible for the inhibition of transcription. However, the results obtained from the oocyte injections corroborate the results obtained from the in vitro experiments using HeLa cell extracts. Accurate transcriptional activity can be restored to the HeLa cell nuclear extracts by the addition of calf thymus RNA polymerase II that had been immunoaffinity-purified (Fig. 7). This argues for a specific effect of the mAb on the RNA polymerase II.

Bona et al. (1982) microinjected antibodies prepared against Drosophila RNA polymerase II into X. laevis oocytes and examined the effect of these antibodies on lambrush chromosomes. Among these antibodies was a monoclonal antibody that seemed to react with both of the largest subunits of the Drosophila enzyme (Krämer et al., 1980); thus, the epitope specificity of this antibody was not established. Several investigators have also injected antibodies to other proteins into oocytes (Fradin et al., 1984; Scheer et al., 1984). However, this technique has not been greatly exploited. The data presented in this paper indicate that oocyte injection is a useful system for examining the effect on specific gene expression of well characterized antibodies prepared against components of the transcription machinery.

Recently, we have injected the peptide-BSA conjugate into Xenopus oocytes and have found that it inhibits transcription from the human H2b gene at a concentration of 400 ng per nucleus; glutaraldehyde-treated BSA at the same concentration did not affect transcription. The peptide-BSA conjugate

FIG. 8. Effect of antibody 3WG2 on transcription of the human genes in X. laevis oocytes. Stage 6 oocytes were coinjected with a plasmid containing either the U1 maxigene (A) or the histone H2b gene (B) and the designated amount of 3WG2 antibody or control mAb (28.13.3s). A plasmid containing the 5 S maxigene was also coinjected to serve as an internal control for successful nuclear injections. The 5 S maxigene transcript does not appear on B because of the extreme difference in size between the H2b and the 5 S maxigene transcripts. Marker lane (first lane on each gel) contained DNA markers (pBR322 digested with MspI and end-labeled with 32P).

did not inhibit the transcription of RNA polymerase I or III transcripts in oocytes (data not shown).

Conclusion—While a precise role for the C-terminal domain cannot be derived from these data, our results indicate that some critical interaction of RNA polymerase II with the rest of the transcription complex is disrupted by the mAbs and by the synthetic peptide that reacts with these mAbs. This interaction might be with specific transcription factors or with the general topography created by proteins that are bound to the promoter region. This interaction might be more critical for certain promoters or might be more important when RNA polymerase II is not in excess.

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Addendum—While this paper was being reviewed, W.-Y. Kim and M. E. Dahmus (1989) showed that the Ad2 MLP could be accurately transcribed in a reconstituted HeLa cell transcription system by calf thymus RNA polymerase II that lacked the C-terminal domain.

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