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The C-Terminal Domain of the Largest Subunit of RNA Polymerase II and Transcription Initiation

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Monoclonal antibodies specific for the evolutionarily conserved C-terminal heptapeptide repeat domain of the largest subunit of RNA polymerase II inhibited the initiation of transcription from mammalian promoters in vitro. Since these antibodies did not inhibit elongation and randomly initiated transcription, the heptapeptide repeats may function by binding class II transcription initiation factor(s).

The largest subunit polypeptide (RPO21) of eucaryotic RNA polymerase II contains an unusual C-terminal domain composed of tandemly repeated copies of the consensus heptapeptide sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser (3, 4, 10, 23, 25). A monoclonal antibody that recognizes a determinant on RNA polymerase II_A but not on polymerase II_B (9) inhibited in vitro transcription by polymerase II from several promoters but had no effect on randomly initiated transcription of a calf thymus DNA template (11). The subsequent demonstration that the conversion of polymerase II_A to the II_B form is the result of proteolysis of the C-terminal domain from the RPO21 polypeptide (3, 10) implied that the anti-polymerase II_A monoclonal antibody recognized the C-terminal domain of the RPO21 polypeptide and that this domain might therefore play a special role in promoter-directed transcription. In this study, we have used monoclonal antibodies with a known specificity for the RPO21 heptapeptide repeats to provide further support for the view that this domain of polymerase II has an important function in the initiation of promoter-directed transcription.

Anti-heptapeptide-repeat monoclonal antibodies. A 37-residue synthetic peptide (Arg-[Ser-Pro-Ser-Tyr-Ser-Pro-Thr]$_5$-Gly-NH_2) (Alberta Peptide Institute) containing five of the consensus heptapeptide repeats of the RPO21 polypeptide was coupled to albumin with 1% glutaraldehyde and used to raise monoclonal antibodies in BALB/c mice by using techniques described previously (21). With solid-phase radioimmunoassays, we identified four hybridoma clones, Jel 351, Jel 352, Jel 354, and Jel 355, which secreted antibodies that reacted strongly with the 37-mer antigen (data not shown). Three of these monoclonal antibodies also recognized a determinant on a single polypeptide with same apparent molecular weight as the nonproteolyzed (II_A) RPO21 polypeptide in *Saccharomyces cerevisiae* and calf thymus RNA polymerase II preparations (Fig. 1). The order of cross-reactivities toward the RPO21 polypeptide antigens was Jel 352 > Jel 354 >> Jel 351 (> Jel 355). In other immunoblotting experiments using both yeast whole-cell (2) and HeLa nuclear (data not shown) extracts, Jel 352 antibodies detected only the RPO21 polypeptide.

Inhibition of promoter-dependent transcription. We tested the effect of the Jel 352 antibodies on in vitro transcription from several different mammalian promoters. When samples of a HeLa cell nuclear extract (12) were preincubated with increasing amounts of Jel 352, transcription from the adenovirus 2 major late promoter (Ad2 MLP), as measured by accumulation of an α-amanitin-sensitive 405-nucleotide-long transcript, was reduced (Fig. 2A). Since a 25-fold molar excess of the 37-mer heptapeptide antigen suppressed the inhibition of Ad2 MLP transcription by Jel 352 (Fig. 2A), antibody binding RNA polymerase II must directly interfere with Ad2 MLP transcription. These monoclonal antibodies also inhibited transcription from two other promoter DNAs,

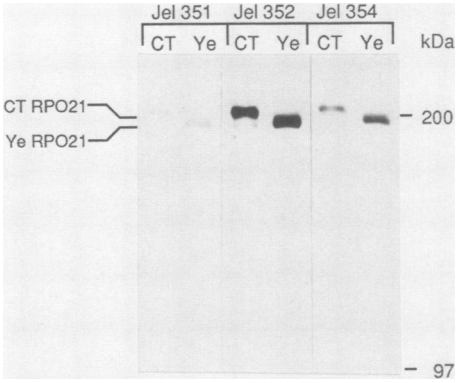

FIG. 1. Immunoblotting of RNA polymerase II with anti-37-mer monoclonal antibodies. Subunit polypeptides of purified yeast (Ye) and calf thymus (CT) RNA polymerase II (0.5 μg) were fractionated on a 5% sodium dodecyl sulfate-polyacrylamide gel, electrophoretically transferred to nitrocellulose, and probed with a 1:5,000 dilution of 2-mg/ml solutions of the anti-37-mer monoclonal antibodies Jel 351, Jel 352, and Jel 354 by using an alkaline phosphatase conjugate method described by the manufacturer (Bio-Rad Laboratories). The positions of the nonproteolyzed (II_A) form of the RPO21 polypeptides as determined by Coomassie blue staining (data not shown) are indicated. Both the calf thymus (16) and yeast (6) RNA polymerase II preparations contained considerable amounts of the proteolyzed (II_B) form of the RPO21 polypeptide which was not detected with these antibodies. Prestained high-molecular-weight standards (Bethesda Research Laboratories, Inc.) were used as size markers. kDa, Kilodaltons.

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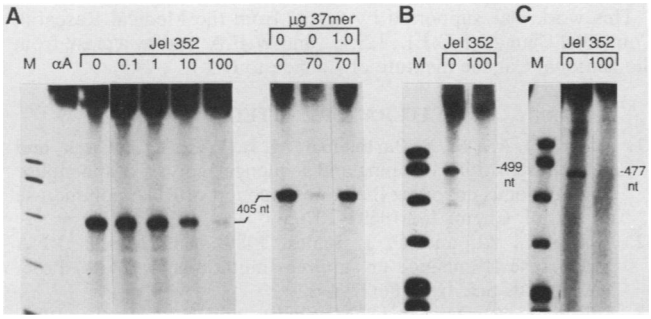

FIG. 2. Inhibition of accurate runoff transcription from mammalian class II promoters by anti-37-mer monoclonal antibodies. Accurate runoff transcription assays in vitro were performed as described by Burton et al. (7), and the reaction products were separated on 5 M urea–5% polyacrylamide gels. The dried gels were then exposed to X-ray film with an intensifying screen. Anti-37-mer monoclonal antibodies in 2.0 μl were preincubated for 10 min at 25°C with 10 μl of the HeLa cell nuclear extract before the addition of 8 μl of a mix containing $MgCl_2$, template DNA, and ribonucleotides. Antibody concentrations (in micrograms per milliliter) in the final incubation are as indicated above the lanes. (A) Runoff transcription at 30°C from the Ad2 MLP DNA. The lane marked αA shows that the 405-nucleotide runoff transcript is sensitive to 1 μg of α-amanitin per ml. For the lanes on the right, Jel 352 antibodies were preincubated for 10 min with the indicated amount (in micrograms) of the 37-mer peptide before addition of the nuclear extract. (B) Transcription of the 5'-deleted (to −34) Ad2 MLP DNA. (C) Transcription of the murine dihydrofolate reductase promoter DNA. Size markers (lanes M) were pBR322 DNA restricted with *Hpa*II and 3' end labeled with [α-^{32}P]dCTP. For transcription from the wild-type and mutant Ad2 MLP DNAs, reactions were done at 30°C with 12 mM $MgCl_2$ and either *Hin*cII pMLP1 DNA (7) or *Bst*XI-restricted pSVBIA34 DNA (22) (30 μg/ml). For the dihydrofolate reductase promoter, transcription was performed at 25°C with 4 mM $MgCl_2$ and *Pvu*II-restricted pSS625 DNA (14) (20 μg/ml).

a mutant Ad2 MLP (Fig. 2B) which lacks the binding sites for the major late transcription factor (8, 22) and the mouse dihydrofolate reductase gene (Fig. 2C), which has binding sites for the transcription factor Sp1 but lacks a TATA sequence (13).

No inhibition of randomly initiated transcription. To determine whether the anti-37-mer antibodies were interfering with an essential function of RNA polymerase II, such as DNA binding or ribonucleotide polymerization, we examined the effect of the Jel 352 antibodies on randomly initiated transcription. The presence of Jel 352 antibodies, at a concentration (100 μg/ml) that almost completely abolished transcription from the mammalian promoter DNAs, had no influence on random transcription of calf thymus DNA (Fig. 3).

Initiation, not elongation, of transcription is inhibited. To determine which step of promoter-dependent transcription is blocked by the anti-37-mer monoclonal antibody, we used a protocol (15) illustrated in Fig. 4A. Jel 352 antibodies had no effect on transcription when added during transcription elongation (t = 42 and 52 min), whereas the antibody completely inhibited polymerase II transcription when added either before or within a few minutes after the addition of template DNA to the HeLa cell nuclear extract (Fig. 4B). Increasing the time that the HeLa cell nuclear extract was preincubated with template DNA before the addition of antibody reduced the inhibitory effect of this reagent. The behavior of the monoclonal antibody Jel 352 therefore differed somewhat from that described recently by Laybourn and Dahmus (19) for their G7A5 monoclonal antibody. While both Jel 352 and G7A5 inhibited initiation of transcription, G7A5 also affected the elongation phase of transcription, albeit at a 10-fold-higher concentration of antibody, a concentration which also affects transcription by polymerase III (15). In other experiments (M. Moyle and C. J. Ingles, unpublished data), the 37-mer peptide itself (at 2 to 4 mg/ml) also blocked initiation of promoter-dependent transcription in vitro. However, since a number of unrelated peptides also inhibited transcription initiation at similarly high concentrations, the significance of the inhibition by short heptapeptide repeat analogs is unclear.

Our finding that the anti-heptapeptide-repeat monoclonal antibodies block the initiation of transcription by polymerase II but do not block elongation of correctly initiated transcription or randomly initiated transcription in vitro formally confirms the original interpretation (3, 10) of the results reported by Dahmus and Kedinger (11; see also reference 19). Our data therefore provide some support for the suggestion that the tandemly repeated heptapeptide domain of polymerase II participates in the initiation of transcription by polymerase II at bona fide promoters (1, 3, 10). Since this antibody inhibited transcription from three promoters with different requirements for DNA-binding transcription factors, the C-terminal domain may interact with general class II transcription factor(s). Alternatively, the bound anti-C-terminal domain antibodies may prevent access of an essential initiation factor to the enzyme-DNA preinitiation complex. Since both Zehring et al. (25) and Kim and Dahmus

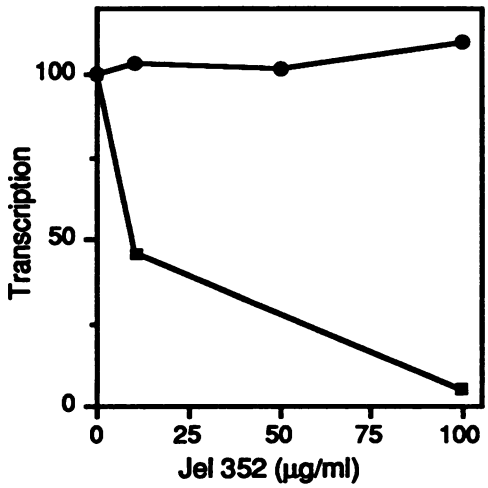

FIG. 3. Anti-37-mer monoclonal antibodies inhibit transcription of the Ad2 MLP DNA but not randomly initiated transcription of calf thymus DNA. Transcription reactions each contained 0.05 M Tris (pH 7.9), 7 mM NaF, 1.8 mM $MnCl_2$, 0.8 mM each GTP, CTP, and ATP, 1.2 μM UTP, 0.38 mg of calf thymus DNA per ml, 3 mM 2-mercaptoethanol, 75 mM ammonium sulfate, and 5 μCi of [5,6-^{3}H]UTP (41.7 Ci/mmol). The anti-37-mer monoclonal antibody Jel 352 in 2 μl was preincubated with 20 μl of HeLa cell nuclear extract for 10 min at room temperature. To initiate transcription, 25 μl of a cocktail containing the other reagents was then added. Transcription by RNA polymerase II of calf thymus DNA (●) was that portion of the total incorporation of [5,6-^{3}H]UMP that was inhibited by 1 μg of α-amanitin per ml and is expressed as a percentage of [5,6-^{3}H]UMP incorporated by polymerase II in the absence of monoclonal antibody. Values plotted are averages of two determinations. In the absence of antibody, RNA polymerase II incorporated 1.8 pmol of [^{3}H]UMP. Data for the transcription of Ad2 major late DNA (■) were quantitated by densitometric scanning of the experiment shown in Fig. 2A, and datum points are expressed as percentages of transcription in the absence of the monoclonal antibody.

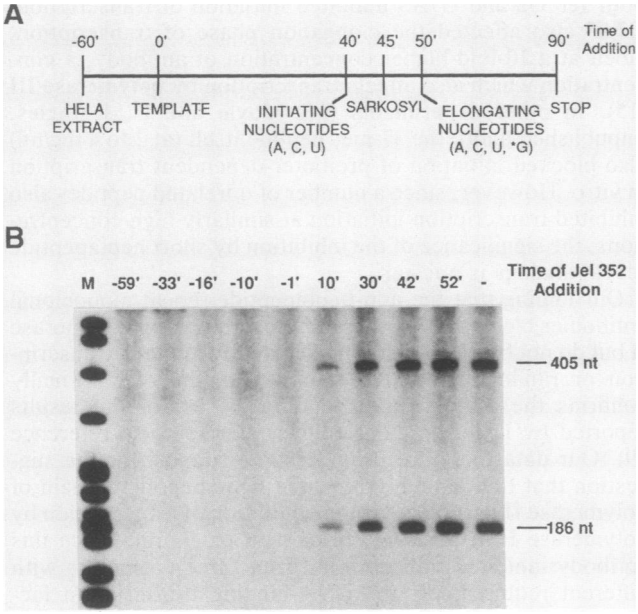

FIG. 4. Inhibition of transcription initiation by the anti-37-mer monoclonal antibody Jel 352. (A) Reaction scheme. At t = 0 min (0'), 4 μl of template cocktail that contained pMLP1 DNA (170 ng/μl) was added to 14 μl of HeLa cell nuclear extract in 17 mM $MgCl_2$ and 4 mM EGTA [ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]. At t = 40 min, 2 μl of initiating cocktail containing 1 mM each ATP, CTP, and UTP was added to the reaction mixture. Five minutes later, 2 μl of 4% Sarkosyl was added, and at t = 50 min 5 μl of elongating cocktail that contained 3.2 mM each ATP, CTP, and UTP, 150 μM GTP, and 5 μCi of [α-^{32}P]GTP was added. Reactions at 30°C were stopped 40 min later. At the time points indicated above the lanes, the anti-37-mer monoclonal antibody Jel 352 (final concentration, 100 μg/ml) was added to these transcription reactions in a volume of 1 μl. In the presence of Sarkosyl, runoff transcription from the Ad2 MLP was approximately equally divided between the expected 405-nucleotide (nt) transcript and a 186-nucleotide prematurely paused or terminated transcript (15). M, Size markers.

(18) have reported that the repeated heptapeptide domain is apparently not required for promoter-dependent transcription in vitro, its actual role is uncertain. However, their reconstituted transcription systems may bypass a requirement for the C-terminal domain of the RPO21 polypeptide.

In addition to possible interactions with a general class II transcription factor, we (2, 4, 17) and others (1, 5, 24) have previously suggested that the C-terminal domain could mediate recruitment of RNA polymerase II to promoters via direct contacts between the heptapeptide domain of polymerase II and the acidic activation domains of DNA-binding transcription factors. Indeed, genetic evidence from our laboratory is consistent with the idea that yeast DNA-binding transcription factors such as GAL4 and GCN4 might interact with the C-terminal domain. Phosphorylation of this domain of polymerase II may then be required to initiate transcription (19, 20, 24). Precise knowledge of the variety of molecular interactions involving this novel domain of polymerase II may be essential to understanding mechanisms of transcription initiation.

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