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Evidence that a triplex-forming oligodeoxyribonucleotide binds to the c-*myc* promoter in HeLa cells, thereby reducing c-*myc* mRNA levels

(transcription/DNase I hypersensitivity/triplex)

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ABSTRACT **A synthetic 27-base-long oligodeoxyribonucleotide, termed PU1, has been shown to bind to duplex DNA to form a triplex at a single site within the human c-*myc* P1 promoter. PU1 has been administered to HeLa cells in culture to examine the feasibility of influencing transcription of the c-*myc* gene *in vivo*. It is shown that uptake of PU1 into the nucleus of HeLa cells is efficient and that the compound remains intact for at least 4 hr. In nuclei extracted from PU1-treated cells, inhibition of DNase I cleavage is detected within the c-*myc* P1 promoter at the target site for triplex formation. The inhibition is shown to be both site and oligodeoxyribonucleotide specific. After cellular uptake of PU1, it is shown that steady-state mRNA arising from the c-*myc* P1 initiation site is selectively reduced relative to total mRNA, relative to mRNA from the alternative c-*myc* P2 initiation site, and relative to mRNA derived from the β-actin promoter. Significant mRNA repression is not seen upon treating cells with oligodeoxyribonucleotides that fail to bind to the P1 promoter target. Taken together, these data suggest that triplex formation can occur between an exogenous oligodeoxyribonucleotide and duplex DNA in the nucleus of treated cells.**

The use of oligodeoxyribonucleotides (ODNs) that bind and inactivate cellular mRNA by an antisense interaction is now well documented in a variety of systems (1–6), including c-*myc* (1–4). Studies have also been initiated to explore ODNs with the potential to bind to duplex DNA to form a triple helix (7–11). Although in an early stage, work from several laboratories has made it possible to design ODNs that have the capacity to bind to purine-rich duplex targets *in vitro* with good site selectivity (7–14).

The first study of transcription inhibition by triplex formation was described 23 years ago for homopolymers, using an *in vitro Escherichia coli* system and RNA as the third strand (15). More recently, it was shown (7) that a synthetic 27-base-long ODN, termed PU1, can bind tightly to duplex DNA at a single site within the human c-*myc* promoter at physiological pH (see Fig. 1). In the same study it was shown that PU1 can inhibit transcription of the c-*myc* gene in a cell-free assay system (7). Here, we present further evidence demonstrating the specificity of triplex formation by PU1 and present data that suggest that PU1 can repress transcription of the c-*myc* gene in HeLa cells, following uptake and triplex formation in the nucleus.

EXPERIMENTAL PROCEDURES

Synthesis and Characterization of ODNs. Unmodified ODNs were synthesized by the phosphoramidite method (MilliGen/Biosearch, Milford, MA) and purified by C_{18} HPLC chromatography. Subsequent to detritylation and exclusion chromatography on Sephadex G-25 resin, the purity of the resulting ODNs was confirmed by ion-exchange HPLC and by electrophoresis. ODNs were freeze-dried and then redissolved in distilled water (for binding analysis) or growth medium (for *in vivo* studies). ^{32}P 5′-end-labeled ODNs were prepared by standard procedures (16), followed by Sephadex G-25 exclusion chromatography to remove unincorporated label.

Cell Growth and ODN Treatment. HeLa cells were grown in suspension culture in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 10% glutamine (GIBCO). Viable cells were counted by the trypan blue dye-exclusion method. Exponentially growing cells were concentrated by centrifugation at 6000 × *g* for 8 min, added to an equal volume of ODN in the same medium, and incubated at 37°C with gentle rolling. At the times indicated, samples were removed, chilled on ice, and diluted with 10 volumes of ice-cold medium without serum. After sedimentation, the cell pellet was washed two more times with 10 volumes of medium, and the final pellet was subjected to isotonic Nonidet P-40 lysis (10 mM Tris·HCl/3 mM $MgCl_2$/10 mM NaCl/0.5% Nonidet P-40, pH 7.8). Nuclear and cytoplasmic fractions from the cell lysates were prepared by standard procedures (16, 17). Cytoplasmic RNA was purified by protease K and SDS treatment of the supernatant, followed by organic extractions and ethanol precipitation. The resultant RNA pellet was resuspended in diethyl pyrocarbonate-treated water, quantified by absorbance at 260 nm, and stored at −80°C.

To halt RNA synthesis and to determine the mRNA half-life, cells were treated with actinomycin D (United States Biochemical) at 5 μg/ml for the indicated times prior to sampling and RNA analysis.

DNase I Hypersensitive Site Analysis. Nuclei (4×10^6), harvested from HeLa cultures treated with ODN for 2 hr were resuspended in 0.5 ml of ice-cold digestion buffer (20 mM Hepes, pH 7.9/10 mM $MgCl_2$/4 mM $CaCl_2$/20% glycerol). After transfer to 22°C, the nuclei were immediately treated with 0–100 units of DNase I (Promega) per ml for 3 min. The reaction was terminated by the addition of 50 μl of 5% SDS/100 mM EDTA. DNA was subsequently purified, treated with *Xba* I, purified again, and electrophoresed in 1.1% agarose. DNA was transferred onto a nylon membrane (Zetabind; Cuno), probed, and washed according to the manufacturer's recommendations. The probe was a 657-base-pair (bp) *Pvu* II/*Xba* I fragment (from 522 to 1179), labeled by random oligonucleotide labeling (Prime-It kit; Stratagene).

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Abbreviation: ODN, oligodeoxyribonucleotide.

Quantification of Steady-State mRNA Levels. For each data point, 10 μg of total cytoplasmic RNA was hybridized to the appropriate complementary RNA probe and analyzed by the RNase protection method as described (18). The protected RNA from P1 or P2 start sites yields 512- and 342-base fragments, respectively. The human β-actin probe (a gift of L. S. Chang, Princeton University) was obtained by linearization (with *Hph* I) of pGEMβ-actin (the *Sca* I/*Eco*RI fragment from pT3β-actin cloned between *Sma* I and *Eco*RI), yielding a probe of 279 nucleotides and a protected fragment of 86 bases. All linearized probes were transcribed with T7 polymerase (BRL).

RESULTS

Selectivity of PU1 Binding to the c-*myc* Target Duplex. Footprinting analysis of PU1 binding to purified DNA fragments has shown that it can form a site-selective triple helix at a single target site roughly −135 relative to the P1 cap site of the human c-*myc* promoter (7). In the analysis of triplex formation in that previous study, it was assumed that PU1 binding occurred as a parallel triple helix. However, subsequent work has shown that PU1 and its homologues prefer to bind antiparallel with respect to the −135 target (19). For PU1, this antiparallel orientation gives rise to an AGC and a GAT triplet mismatch, which are identified by underlining in Fig. 1.

To determine if PU1 binding to the −148/−122 target site is specific enough to be used in a biological context, triplex formation at pH 7.8 and 37°C was measured by titrating dilute radiolabeled PU1 (at 10^{-10} M) with an unlabeled restriction enzyme digest of plasmid DNA bearing the duplex DNA target site as a 479-bp fragment (Fig. 2). As seen, binding is readily detected as comigration with the 479-bp fragment; the resulting titration saturates near 10^{-7} M of added duplex target, in good agreement with a dissociation constant of 5×10^{-7} M, which was measured previously with a short synthetic duplex fragment (7). At the highest added duplex concentration, binding cannot be detected elsewhere within the 6000-bp plasmid digest, thereby confirming the apparently high site selectivity for triplex formation by PU1 at the −148/−122 site. When similarly analyzed, binding of the control oligonucleotides PY1 and HIV31a (Fig. 1) could not be detected (data not shown).

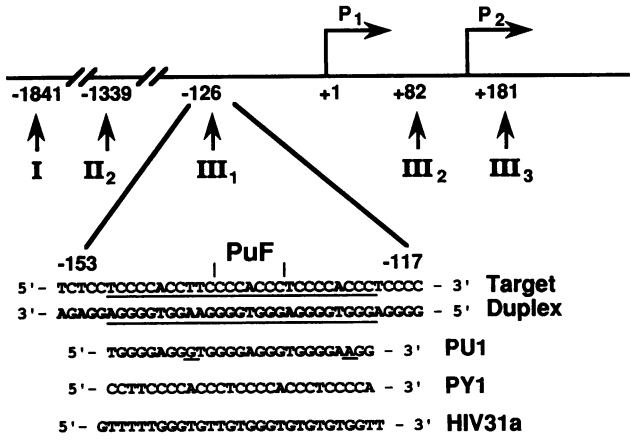

FIG. 1. Map of the human c-*myc* gene showing the two major c-*myc* promoters P1 and P2. The sequence of the putative triplex-forming region (7) from −148 to −122 is indicated, as is the sequence of PU1 and the control ODNs PY1 and HIV31a. Underlined bases in the duplex target refer to the span of the ODN binding domain, as assessed by DNase I footprinting on cloned DNA fragments (7). The two underlined bases in PU1 correspond to what are believed to be mismatches arising in the preferred antiparallel orientation for the bound complex (ref. 19; M.E.H., unpublished data). The transcription factor PuF binding site is also indicated, which spans sites −137/−131 (18). Arrows point to the positions of major DNase I cleavage sites: that for site III_1 is centered at position −126 (20, 21). All numbering is relative to the P1 promoter.

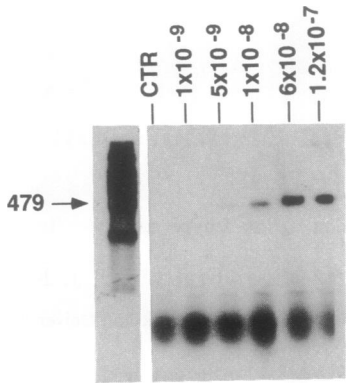

FIG. 2. Site-selective binding of PU1 to the c-*myc* gene. The plasmid pMHX (22) was digested with *Pst* I and *Taq* I, thereby liberating the −148/−122 target site at the center of a 479-bp fragment. The remainder of the plasmid exists as a series of 9 unrelated fragments. The marker lane to the far left corresponds to that series of 10 fragments, radiolabeled with ^{32}P. Binding was performed by incubation of ^{32}P-labeled PU1 (at 10^{-10} M) at 37°C for 1 hr with increasing concentrations of the pMHX digest in the standard binding buffer (20 mM Tris·HCl/1 mM spermine HCl/10 mM $MgCl_2$/10% sucrose, pH 7.8). Added plasmid DNA concentration (indicated above each lane) was calculated in terms of moles of binding site (plasmid molecule) equivalents per liter. Triplex formation was visualized by observing the comigration of the ^{32}P-labeled ODN with the 479-bp target duplex in a 5% acrylamide gel at room temperature, in the presence of the standard Mg^{+2}-containing electrophoresis buffer (7). CTR, control in which plasmid was not added.

Subsequent to the initiation of studies described here, it has been shown that the affinity and site selectivity of PU1 homologues can be significantly improved by means of selective base substitution in the context of an antiparallel binding model (ref. 19; M.E.H. and D.J.K., unpublished work). However, the data shown in Fig. 2 and previous work (7, 19) suggest that PU1 possesses sufficient duplex sequence selectivity at 37°C and pH 7.8 to be used as an initial candidate for the study of triplex formation in HeLa cells.

Uptake and Stability of PU1 in Cultured HeLa Cells. ODN transport into cells is now well documented (1, 23), although published data on nuclear uptake are scarce. To measure uptake by HeLa cells directly, cultures were exposed to ^{32}P 5'-end-labeled ODNs and then incubated for increasing periods of time. The kinetics and intracellular distribution of PU1 are shown in Fig. 3*A*. Both the cytoplasmic and nuclear uptake of ^{32}P increased in an exponential fashion. Nuclear uptake appeared to reach a steady state by 2 hr, whereas cytoplasmic uptake continued. Control ODNs PY1 and HIV31a yielded similar uptake rates and endpoints in this assay (data not shown), indicating that nuclear uptake is not measurably sequence dependent in HeLa cells. Similar ODN uptake rates by HeLa cells have been observed by others (2).

To evaluate the physical state of the ODN in the nuclear and cytoplasmic fraction, material was analyzed by high-resolution electrophoresis. To visualize the unaltered fragment profile, samples were derived from an equal number of cells, without chemical extraction of any kind. Over 4 hr, degradation of PU1 could not be detected in the medium (Fig. 3*B*, lanes 1 and 2), the nucleus (Fig. 3*B*, lanes 3–7), or cytoplasm (Fig. 3*B*, lanes 8–12). As shown, the quantity of full-length PU1 associated with the cytoplasmic fraction at each time point is roughly twice that associated with the nuclei, in good agreement with the 2-fold difference in overall

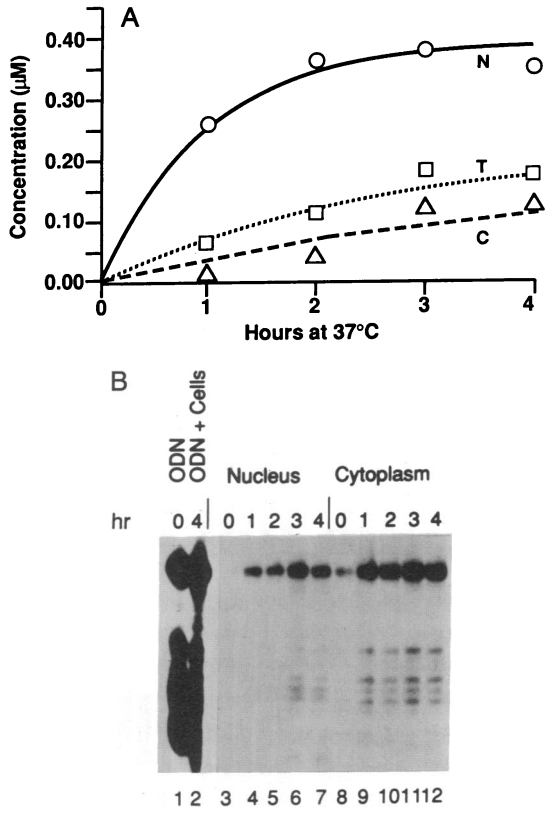

FIG. 3. (*A*) Cellular uptake of ^{32}P-labeled PU1. HeLa cells at 1.6 × 10^7 cells per ml were incubated at 37°C with ^{32}P-labeled PU1 at a final concentration of 0.1 μM for 4 hr. Samples were removed hourly and fractionated into nuclear and cytoplasmic components. The ODN concentration in each compartment was determined by scintillation counting of 10-μl samples. Based on the relative volume of HeLa cells and their nuclei (24, 25), the apparent intracellular concentration of PU1 was calculated and plotted vs. time of incubation at 37°C. Curves correspond to an empirical fit to a simple exponential. N, PU1 in nuclei; C, PU1 in cytoplasm; T, total PU1 in cells. (*B*) Stability of PU1 in cell fractions. Ten-microliter samples from nuclei (lanes 3–7), cytoplasm (lanes 8–12), or extracellular medium (lanes 1 and 2) were freeze-dried and analyzed on a 12% sequencing gel. Lanes 1 and 2 compare the effects of no incubation and a 4-hr incubation of ODN in the cell culture medium. Cell viability was not affected over the 4-hr period.

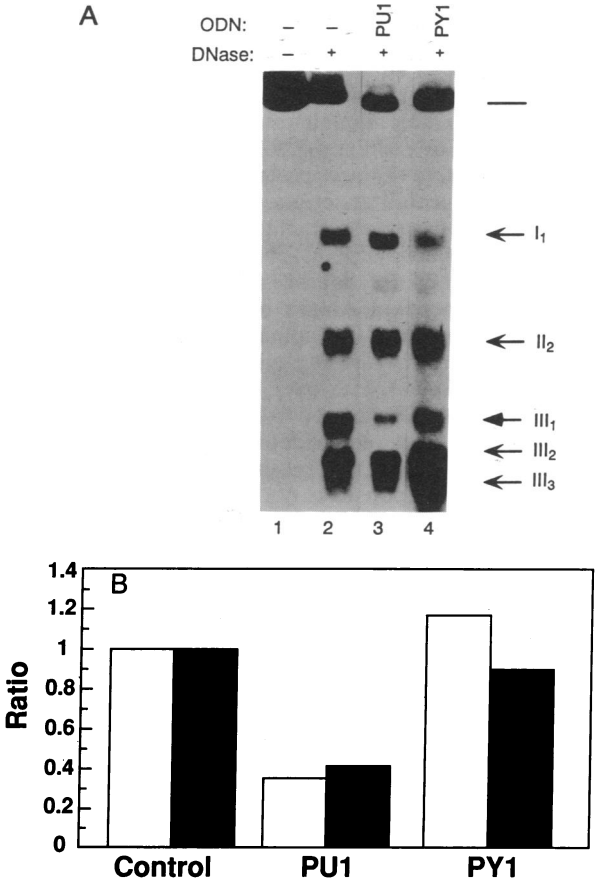

FIG. 4. DNase I hypersensitive site analysis of chromatin from HeLa cells treated with ODNs in culture. (*A*) A representative Southern blot is presented of DNase I-digested nuclei from control and ODN (125 μM)-treated cells, near the midpoint of a series of DNase I titrations (30 units/ml). The bar on the right indicates the position of the uncleaved restriction fragment, and arrows point to previously characterized DNase I hypersensitive sites (see Fig. 1). (*B*) Densitometry of DNase I hypersensitivity analyses. Values obtained from autoradiograms from two independent experiments at 125 μM ODN were averaged. The ratio of cleavage at site III_1 relative to that at site I (white bars) or at site II_2 (black bars) have been cataloged for untreated control or PU1- or PY1-treated cells.

mass uptake per cellular compartment, as detected by scintillation counting (Fig. 3*A*).

PU1 Binding in the Nucleus, as Assessed by DNase I Protection. The duplex complement of the PU1 triplex becomes resistant to DNase I cleavage *in vitro*, a property that has been employed for high-resolution mapping of triplex formation on cloned DNA fragments (7, 19). We have used such resistance to DNase I cleavage as the basis for a method to map binding of PU1 in the nucleus.

In chromatin from several human cell lines, including HeLa, c-*myc* displays a distinct set of DNase I hypersensitive sites (20, 21). Two of these sites, II_2 and III_1, have been associated with promoter function (21). Based upon high-resolution analysis, Siebenlist *et al.* (20) have determined that III_1 spans approximately a 40-bp domain centered at site −126 (Fig. 1). Consequently, hypersensitive site III_1 is coincident with the target site for triplex formation by PU1, suggesting that if site-selective triplex formation were to occur in the nucleus, the resulting PU1 triplex might be protected from DNase I digestion, under conditions where adjacent sites I, II_2, and III_2 would be unaffected.

In the DNase I assay (Fig. 4*A*), nuclei were isolated from HeLa cells after 2.5 hr of incubation with PU1 or a control ODN, PY1, then treated with DNase I and analyzed by Southern blotting. In the absence of ODN treatment (Fig. 4*A*, lane 2) or upon treatment with the PY1 control (Fig. 4*A*, lane 4), DNase I cleavage within the 5′ flanking domain is as described previously for the c-*myc* promoter region (20, 21). However, nuclei extracted from HeLa cells treated with PU1 displayed selective inhibition of DNase I cleavage at site III_1 (Fig. 4*A*, lane 3). Quantification of autoradiograms from two independent experiments indicated a 3- to 4-fold reduction in DNase I cleavage rate at III_1 upon PU1 treatment as compared to flanking sites I and II_2 (Fig. 4*B*).

Although DNase I mapping of this kind must ultimately be corroborated by other methods, to our knowledge, these results provide the first evidence for triplex formation upon a target site in the nucleus of cells treated with an ODN.

Modulation of Steady-State c-myc mRNA Levels *in Vivo*. Based upon the functional importance of the promoter region containing the −148/−122 site (18, 26, 27) and the fact that PU1 binding has been shown to repress c-*myc* transcription in a cell-free HeLa transcription system (7), we wished to determine whether PU1 uptake and binding within the nucleus could similarly modulate c-*myc* transcription in HeLa cells.

Since c-*myc* mRNA species have an extremely short half-life (refs. 28 and 29; see below) and ODN accumulation in HeLa cell nuclei appears to be complete within 2 hr, we chose to monitor triplex-mediated transcription arrest in HeLa cells 2.5 hr after administration of ODN to the medium. The ODN effect on cellular mRNA pools is illustrated in Fig. 5*A*. A quantitative RNase protection assay was used to distinguish between transcripts originating from the two relevant c-*myc* promoters, P1 and P2 (ref. 30; see Fig. 1). The results show that the midpoint for PU1-mediated inhibition of P1 mRNA is near 25 μM, as assessed by quantification of data from four independent RNase protection assays (Fig. 5*B Top*). That midpoint is 10–50 times the dissociation constant for the PU1–c-*myc* triplex at physiological pH *in vitro* (refs. 7 and 19; and above). However, given the complexity of a chromatin target, as compared to cloned duplex DNA and the uncertain relation between added ODN concentration and its activity in the nucleus, we feel that the observed difference in the two dose–responses is not surprising.

As seen in Fig. 5 *A* and *B Top*, the observed repression of P1 mRNA appears to be compound specific, in that the control ODNs PY1 and HIV31a did not produce statistically significant mRNA inhibition over the same concentration range.

Steady-state mRNA synthesis from the c-*myc* P2 promoter was assayed independently, as is possible when employing the RNase protection assay. Direct inspection of autoradiograms (Fig. 5*A*) and quantification of similar data (Fig. 5*B Middle*) show that PU1 is a less efficient repressor of P2 mRNA: 50% inhibition of P2 mRNA at $\approx$125 μM, as compared to 50% inhibition of P1 mRNA at 25 μM. Similar to P1 mRNA, the control ODNs PY1 and HIV31a had no significant effect on steady-state P2 mRNA levels, thereby reaffirming the compound selectivity of the observed mRNA repression.

The available genetic evidence suggests that DNA alteration within the −293/−101 (26) or −349/−101 (27) P1 region elicits significant reduction of P1 promoter function in transient cellular assays (26, 27) under conditions where the same mutations produce a much smaller effect upon transcription from P2 (26). In the context of those genetic data, it is therefore likely that triplex formation upon the −148/−122 site would have a greater effect upon P1 promoter activity *in vivo*. The observation that PU1 is a more potent inhibitor of P1 mRNA than P2 mRNA (Fig. 5) is consistent with this prediction; it provides one additional piece of evidence to suggest that the observed ODN effect is specific and due to triplex formation upon the −148/−122 P1 promoter site.

The RNase protection assay in this study was performed on 10 μg of total cytoplasmic RNA, thereby normalizing the effect of ODN treatment to the RNA pool as a whole. Consequently, the data of Fig. 5 confirm the mRNA selectivity of the observed PU1 effect by a second more general criterion.

As a third indication of mRNA selectivity, we have monitored the effect of the three ODNs upon β-actin mRNA, an unrelated gene control. As shown in the raw data (Fig. 5*A*), or from quantification of multiple experiments (Fig. 5*B Bottom*), neither PU1 nor the two controls elicited a significant effect on actin mRNA.

Additional experimentation is required to completely understand the effect of PU1 and its homologues on mRNA levels *in vivo*, especially the effect on short-lived transcripts other than c-*myc*. In particular, it is certain that genes under c-*myc* control (29, 31) will be secondarily affected by c-*myc*-specific ODN treatment. Indeed, in preliminary results, we have observed small but finite repression of histone mRNA levels after PU1 addition (data not shown), whose stability may be cell cycle and c-*myc* dependent (32).

Explanations Other Than Triplex Formation. The data described thus far are consistent with promoter-specific inhibition of transcription initiation by PU1, resulting from triplex formation at P1 site −148/−122. However, it is important to evaluate other potential explanations.

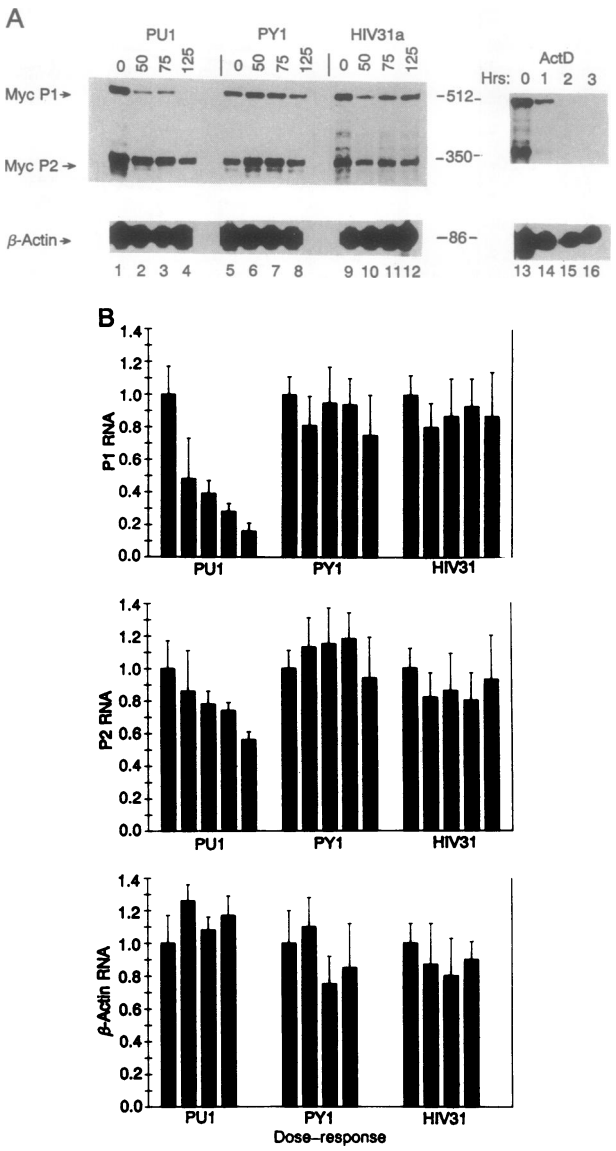

FIG. 5. Cytoplasmic mRNA levels after ODN treatment of HeLa cells. (*A Left*) Autoradiogram of gels showing c-*myc* P1 mRNA, c-*myc* P2 mRNA, and β-actin mRNA. Each time point was generated by analysis of 10 μg of total cytoplasmic mRNA. The size in bases of protected RNA fragments is indicated at the right. The ODN under study was added to 0.5 ml of cell growth medium at 6×10^6 cells per ml. The final medium concentration in μM strand equivalents is displayed at the top of each lane (lanes 1–12). After incubation for 2.5 hr at 37°C, RNA was harvested and analyzed. Cell viability was not affected over the 2.5 hr of ODN treatment. (*A Right*) mRNA lifetimes (lanes 13–16) by the actinomycin D method. Cells (3×10^6), as above, were incubated with actinomycin D. At the times indicated, incubation was terminated, and RNA was analyzed as for ODN-treated cells. (*B*) Autoradiograms as in *A* were quantitated by densitometry. The data are plotted as the measured mRNA level relative to that derived from analysis of mRNA in untreated cells. Error bars refer to one standard deviation of the mean of at least four independent experiments. (*Top*) c-*myc* P1 RNA. (*Middle*) c-*myc* P2 RNA. (*Bottom*) β-Actin RNA. In *Top* and *Middle* the ODN concentrations were (from left to right) 0, 25, 50, 75, and 125 μM. In *Bottom*, the ODN concentrations were 0, 50, 75, and 125 μM.

Widespread transcription inhibition. Formally, it is possible that the observed promoter-specific differences could have resulted from some sort of widespread nonspecific inhibition of transcription initiation by PU1, with measured

differences among different steady-state mRNA levels reflecting the distinct natural decay rates of the various transcripts. However, this explanation of the data demands an ad hoc requirement for high ODN sequence specificity to the presumed effect. Additionally, a widespread inhibition hypothesis requires that the observed inhibition of mRNA levels by PU1 is directly related to the intrinsic decay rates of the mRNAs. Consequently P1, the more sensitive mRNA, should display a shorter intrinsic mRNA lifetime than P2.

To test this prediction, P1 and P2 mRNA levels were measured in the HeLa cell assay system over a 3-hr period following treatment with the nonspecific transcription inhibitor actinomycin D (Fig. *5A Right*). As seen in the autoradiograms, and confirmed by densitometry (data not shown), c-*myc* P2 transcripts display an intrinsic half-life in HeLa cells somewhat *shorter* than that of P1 transcripts. These values are in agreement with previously published values for the two mRNA species (28, 29). Thus, the effect of PU1 on steady-state mRNA levels is *opposite* that expected from the difference in mRNA lifetime and, consequently, is difficult to rationalize in terms of the widespread inhibition model.

Alteration of the mRNA decay rate. The RNase protection assay used above is quantitative and can distinguish between P1 and P2 mRNA species but does not measure transcription rates *per se*. Therefore, the hypothesis that PU1 acts by gene-specific inhibition of transcription initiation has not been addressed definitively. A nuclear run-on assay, which is often used to measure transcription rates, could not be used in this study because it is less sensitive and could not distinguish between transcription inhibition from P1 and P2.

Because transcription initiation is not measured directly by the RNase protection assay, we sought indirect evidence that PU1 treatment alters P1 transcription rather than the characteristic P1 mRNA decay rate (either effect could alter the steady-state mRNA level). The data (not shown) indicate that the rate of change of steady-state P1 and P2 mRNAs in HeLa cells after PU1 addition is identical to the decay kinetics resulting from nonspecific transcription arrest by actinomycin D. Thus, these data provide indirect evidence that the observed reduction of c-*myc* mRNA levels by P1 is occurring at the transcription level, rather than as a result of some unexpected change in steady-state mRNA decay kinetics.

Direct binding of PU1 to protein. It is also possible that the observed PU1-mediated inhibition might have arisen due to binding of PU1 to the PuF factor (18) or some other regulatory protein. Although this possibility is difficult to rule out definitively *in vivo*, we have been unable to detect purified PuF binding to PU1 *in vitro* (E.H.P. and S.J.F., unpublished results). For this reason, the direct protein binding explanation appears unlikely at present.

DISCUSSION

In the present work, evidence is presented to confirm the sequence-selective binding of PU1 to the −148/−122 promoter site at physiological pH and temperature (Fig. 2). It is shown that in the absence of chemical modification PU1 can enter the nucleus of HeLa cells and remains intact for at least 4 hr (Fig. 3).

Evidence is presented to suggest that site-selective triple helix formation can occur in the nucleus, based upon selective inhibition of DNase I cleavage at the predicted site of triplex formation (Fig. 4). It is also shown that at a concentration that leads to selective reduction of DNase I cleavage at the −148/−122 target site in the nucleus, PU1 treatment elicits a 10-fold reduction of P1 c-*myc* steady-state mRNA in HeLa cells. Under the same conditions, the P2 mRNA level is much less effected by PU1, and no effect on β-actin could be detected. In all instances, HeLa cells treated with unrelated ODNs did not show significant effects at the mRNA level (Fig. 5).

Our findings support a model in which, upon entering the nucleus, PU1 binds to its −148/−122 duplex target sequence to form a triple helix, thereby blocking access to PuF (18) or other transcriptional factors. As a result of such competition, mRNA synthesis from the c-*myc* promoter is selectively inhibited.

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