well involve nested head-to-tail DNA complexes.

ACKNOWLEDGMENTS

We thank Wesley Sundquist and Dan Frantz for stimulating discussions.

Registry No. T_9G_3, 137647-85-9; $T_{12}G_3$, 137741-96-9; T_8G_3T, 137647-86-0; $T_{11}G_3T$, 137741-93-6; $T_8G_6T_8$, 137741-94-7; $T_{12}G_6T_{12}$, 137741-95-8; Na, 7440-23-5; K, 7440-09-7; Rb, 7440-17-7.

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Analysis of Promoter-Specific Repression by Triple-Helical DNA Complexes in a Eukaryotic Cell-Free Transcription System†

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Received June 19, 1991; Revised Manuscript Received September 4, 1991

ABSTRACT: A site-specific triple-helical DNA complex has previously been shown to inhibit DNA binding by eukaryotic transcription factor Sp1. To examine the functional consequences of such inhibition, homopurine target sequences for oligonucleotide-directed triple-helix formation were inserted in various configurations relative to Sp1 transcription activator binding sites, upstream of the TATA element of recombinant eukaryotic promoters. The resulting promoters were tested for activity in the presence or absence of recombinant human Sp1 in a *Drosophila* in vitro transcription system lacking endogenous Sp1. When triple-helical complexes were assembled on the promoters by incubation with specific oligodeoxyribonucleotides, promoter-specific repression of basal transcription was observed in the absence of Sp1. Transcriptional repression required the preassembly of triple-helical complexes before addition of nuclear extract. The degree of basal repression was a function of the number and proximity of triple-helical complexes relative to the basal promoter complex. Repression did not result from triple-helix-induced template degradation. Addition of recombinant Sp1 did not cause derepression. These results suggest that triple-helical complexes can repress transcription primarily by blocking promoter DNA assembly into initiation complexes rather than by occluding Sp1 binding. One of several plausible mechanisms for triple-helix-induced repression involves changes in DNA flexibility. Evidence in favor of this model is provided by a permutation-dependent gel mobility assay in which formation of site-specific triple-helical complexes is shown to stiffen double-helical DNA.

Regulation of transcription initiation from specific promoters is a critical step in the spatial and temporal control of eukaryotic gene expression. The rate and precise sequence specificity of transcription initiation by RNA polymerase II result from the interplay of (i) various cis-acting DNA sequence elements located both proximal (basal promoter elements) and distal (upstream activator binding sites, enhancers) relative to the initiation site; (ii) trans-acting transcription activators and repressors, typically proteins, some of which bind cis-acting promoter elements sequence specifically; and (iii) the DNA double helix itself, which provides a context for local and higher order macromolecular interactions. This interplay serves to regulate the organization of a nucleoprotein initiation complex with RNA polymerase II in a process that is, at present, poorly understood (Saltzman & Weinmann, 1989; Mitchell & Tjian, 1989; Levine & Manley, 1989).

Oligonucleotide-directed DNA triple-helix formation, wherein either a pyrimidine (pyrimidine motif) or a purine (purine motif) oligonucleotide binds to a homopurine double-helical DNA target, is a general approach for sequence-specific DNA recognition (Moser & Dervan, 1987; Doan et al., 1987; Cooney et al., 1988; Beal & Dervan, 1991). Intermolecular triple-helical complexes have been shown to inhibit sequence-specific DNA binding proteins (Maher et al., 1989; Hanvey et al., 1989; François et al., 1989). Kinetic and thermodynamic analyses of oligonucleotide-directed DNA

† Supported by research grants from the National Institutes of Health to P.B.D. (GM-35724) and to B.W. (AR-40780) and the Beckman Institute of the California Institute of Technology to B.W. and an American Cancer Society Postdoctoral Fellowship to L.J.M.

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0006-2960/92/0431-70$03.00/0

triple-helix formation have also been reported (Maher et al., 1990; Plum et al., 1990). Oligonucleotide-directed DNA triple-helix formation therefore represents a promising strategy for creating sequence-specific artificial regulators of DNA function that may be useful in vitro or in vivo (Cooney et al., 1988; Dervan, 1990; Maher et al., 1991).

We wished to analyze the effect of pyrimidine motif triple-helical complexes positioned upstream of the TFIID binding site (TATA box) on transcription initiation from eukaryotic promoters in a cell-free system. Transcription factor Sp1 is a DNA binding protein that recognizes GC-rich sequence elements and activates transcription from a variety of vertebrate cellular and viral promoters [Courey et al. (1989) and references therein]. Sequence-specific triple helices have been shown to inhibit DNA binding by Sp1 as well as other DNA binding proteins including restriction endonucleases and methylases (Maher et al., 1989; Hanvey et al., 1989; François et al., 1989; Collier et al., 1991; Strobel & Dervan, 1991). This observation suggested that formation of triple-helical complexes overlapping Sp1 binding sites might repress Sp1-dependent transcription activation. We have therefore created a series of promoters that carry various distributions of Sp1 binding sites and homopurine triple-helix target sites in overlapping and nonoverlapping arrangements upstream from eukaryotic TATA elements. We describe cell-free transcription experiments that examine three questions. First, what are the effects of site-specific triple-helical complexes on basal (Sp1-independent) transcription? Second, what are the effects of triple-helical complexes that overlap Sp1 sites on transcription activation by Sp1? Third, might triple-helical complexes act to repress transcription through mechanisms other than direct transcription factor occlusion? Interestingly, we find that site-specific DNA triple helices can repress transcription even when the complexes do not overlap transcription factor binding sites. These results suggest other possible repression mechanisms including effects on DNA flexibility.

Experimental Procedures

Oligonucleotides. Oligodeoxyribonucleotides were prepared and purified as previously described (Maher et al., 1990).

Transcription Templates. The E4 template series is based on pG_5E4T, herein designated E4-1 (Lin et al., 1988). This plasmid contains adenovirus gene *E4* sequences from −38 to +250 and five copies of a 19 bp[1] synthetic GAL4 binding site placed 21 bp upstream of the *E4* TATA element. This cassette was inserted between the *Bam*HI and *Hin*dIII sites of pGEM3 (Promega, Madison, WI). Templates E4-2 through E4-6 were constructed by inserting one or more copies of the synthetic 40-mer duplex:

```
GATCTGAGAAAGGAGAGAAAAAGGGGCGGGGCATGCATTG
    ACTCTTTCCTCTCTTTTTCCCCGCCCCGTACGTAACCTAG
```

containing a 21 bp homopurine sequence overlapping a canonical Sp1 element (Maher et al., 1989) into the *Bam*HI site 13 bp upstream from the *E4* TATA element in pG_5E4T. Template E4-11 was constructed by insertion of a tandem trimer of the synthetic 40-mer duplex (above) into the *Hin*dIII site at position −170 in pG_5E4T, after filling all recessed termini with DNA polymerase Klenow fragment. Templates E4-7 and E4-8 were constructed by insertion of the synthetic 73-mer duplex:

```
CTAG{TGGGCGGAGTTAGGGGCGGGAT}ACTC-
    {ACCCGCCTCAATCCCCGCCCTA}2TGAG-

                GAGAAAGGAGAGAAAAAGGGG
                CTCTTTCCTCTCTTTTTCCCCGATC
```

containing two copies of GC boxes III and IV from the SV40 early promoter (Courey et al., 1989) and the 21 bp homopurine sequence, in either orientation into the *Xba*I site 19 bp upstream from the *E4* TATA element in pG_5E4T. Templates E4-9 and E4-10 were constructed by first partially filling recessed termini of the synthetic 73-mer duplex (above) using dTTP, dCTP, and DNA polymerase Klenow fragment. The fragment was then inserted into compatible termini in pG_5E4T generated by a similar partial filling reaction with dATP and dGTP after *Hin*dIII cleavage.

The F template series is based on pF1, which contains *Drosophila fushi tarazu* (*ftz*) sequences from −42 to +570 (Dearolf et al., 1989; Laughon & Scott, 1984) cloned between the *Bam*HI and *Sal*I sites of pBluescriptII KS+ (Stratagene, La Jolla, CA). The construct retains the *ftz* TATA element, but excludes all upstream *ftz* transcription control sequences. Templates F2 through F4 were constructed by inserting the synthetic 26 bp duplex:

```
      CGAGAAAGGAGAGAAAAAGGGGTACG
TCGAGCTCTTTCCTCTCTTTTTCCCCATGC
```

between the *Sac*I site of pF1 (48 bp upstream of the *ftz* TATA element) and either the *Bam*HI (17 bp upstream from TATA; F2), the *Spe*I (23 bp upstream from TATA; F3), or the *Xba*I (30 bp upstream from TATA; F4) sites of pF1 after filling recessed termini of the latter sites with DNA polymerase Klenow fragment. The homopurine triple-helix target sites of templates F2 through F4 are therefore spaced 51 bp, 59 bp, and 65 bp upstream of the *ftz* transcription initiation point, respectively. Template E4-6/F1 was constructed by ligating the 600 bp *Kpn*I–*Sac*I promoter fragment of pF1 between the *Kpn*I and *Sac*I sites of template E4-6. This operation places the *E4* promoter from E4-6 and the *ftz* promoter from F1 in convergent orientation, separated by 850 bp.

Permutation Constructs. The construction of plasmid pHW122 has been described (Wu & Crothers, 1984). Permutation constructs B–F were created by inserting one or more copies of synthetic duplexes into polylinker sites in plasmid pCY7 (Prentki et al., 1987). Plasmid pCY7 contains a tandem 375 bp repeat of the initial *Eco*RI–*Bam*HI segment of pBR322. Host strain HB101 was employed for subcloning in order to maximize construct stability. Permutation constructs B and C were prepared by insertion of one or two copies of the synthetic 40-mer duplex (see above) into the *Bgl*II site of pCY7. Permutation construct F was prepared by filling recessed termini of the synthetic 40-mer duplex (see above) with DNA polymerase Klenow fragment, oligomerizing the duplex by blunt end ligation, and inserting six tandem copies of the resulting 44-mer duplex into the *Sma*I site of pCY7. Permutation construct D was prepared by insertion of four tandem copies of the synthetic duplex:

```
GATCCCAATTGAGAAAGGAGAGAAAAAGTTAAGA
    GGTTAACTCTTTCCTCTCTTTTTCAATTCTCTAG
```

into the *Bgl*II site of pCY7. Permutation construct E was prepared by subcloning the 250 bp *Bam*HI–*Hae*III promoter fragment of E4-6 between the *Bgl*II and *Sma*I sites of pCY7.

Sp1. A polypeptide comprising the 516 carboxyl-terminal amino acids of human transcription factor Sp1 (Sp1-516C) was expressed in *Escherichia coli* strain C600 Δ*lon* (Maher et al., 1989) by isopropyl β-D-thiogalactopyranoside induction of plasmid pSp1-516C (Kadonaga et al., 1987). Bacterial

[1] Abbreviations: bp, base pair(s); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES(K^+), potassium *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; SEM, standard error of the mean; Tris-HCl, 2-ammonio-2-(hydroxymethyl)-1,3-propanediol chloride; Tris-OAc, 2-ammonio-2-(hydroxymethyl)-1,3-propanediol acetate.

extracts were prepared as described (Kadonaga et al., 1987, 1988). Sp1-516C was further purified from crude bacterial extracts by sequence-specific DNA affinity chromatography as described (Kadonaga & Tjian, 1986). Purified Sp1-516C (30 ng/μL; the major band upon silver staining after sodium dodecyl sulfate–polyacrylamide gel electrophoresis) was obtained upon dialysis against either buffer Z^e_{150} [25 mM HEPES(K^+), pH 7.5, 20% (v/v) glycerol, 0.1% (v/v) Nonidet P-40, 10 μM $ZnSO_4$, 1 mM DTT, 150 mM KCl] or buffer X_{50} [25 mM HEPES(K^+), pH 7.0, 20% (v/v) glycerol, 0.1% Nonidet P-40, 10 μM $ZnSO_4$, 1 mM DTT, 50 mM KCl].

Drosophila K_c Cell Nuclear Extract. The *Drosophila* nuclear extract was prepared from cultured K_c cells as previously described (Parker & Topol, 1984) with the following modifications. All extraction steps were performed using buffer A [15 mM KCl, 10 mM HEPES(K^+), pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT]. The crude nuclear pellet was first extracted with 0.25 M ammonium sulfate, and extracted proteins were subsequently precipitated by addition of 0.27 g of solid ammonium sulfate/mL of extract. Nuclear extract preparations buffered to pH values of 7.0 or 7.5 were obtained by dialysis for 4 h against buffer Y_{100} [25 mM HEPES(K^+), pH 7.0 or pH 7.5, 100 mM KCl, 10% (v/v) glycerol, 0.1 mM EDTA, 0.1% Nonidet P-40, 1 mM DTT], clarification by 10-min centrifugation at 10000*g*, and freezing of aliquots in liquid nitrogen.

In Vitro Transcription Reactions. Template mixtures (10 μL) containing oligonucleotide (25 pmol), $MgCl_2$ (25 mM), spermidine trihydrochloride (2.5 mM), KCl (50 mM), and supercoiled E4 series and F series templates (125 ng each) were incubated 30 min at 22 °C. Transcription reactions (25 μL) were assembled by addition to the template mixture of buffer Y_{100} (pH 7.0 or pH 7.5; 2.5 μL), purified Sp1-516C or Sp1 buffer (pH 7.0 or 7.5; 5 μL), and nuclear extract (buffered to pH 7.0 or 7.5; 7.5 μL). The final KCl and $MgCl_2$ concentrations in all transcription reactions were 70 and 10 mM, respectively. When the importance of oligonucleotide prebinding was studied, oligonucleotide was withheld until after addition of nuclear extract. Transcription reactions were initiated immediately, or after subsequent incubation intervals by addition of a mixture of the four ribonucleoside triphosphates (0.6 mM each). Transcription reactions were then incubated a further 30 min at 22 °C.

RNA Isolation and Quantitation. Transcription reactions were terminated by addition of 125 μL stop mix [200 mM NaCl, 20 mM EDTA, 1% (w/v) sodium lauryl sarcosinate, 250 μg/mL tRNA]. This mixture was extracted with phenol, and the nucleic acids were precipitated with ethanol and dried. The resulting pellets were resuspended in 5 μL of water, to which was added Tris-HCl, pH 8.0 (10 mM), EDTA (1 mM), KCl (0.2 M), and 100 fmol of each reverse primer (radiolabeled with T4 polynucleotide kinase and [γ-^{32}P]ATP) in a final volume of 10 μL. The *E4* reverse primer (AACACCACTCGACACGGC) binds positions +24 to +41 of *E4* RNA. The *ftz* reverse primer (GTAGCCATATCGGATGTGTAT) binds positions +58 to +78 of *ftz* RNA. Primer binding reactions were heated to 80 °C for 5 min and were then incubated at 37 °C for 30 min. A 25-μL volume of a solution containing Tris-HCl, pH 8.3 (20 mM), $MgCl_2$ (10 mM), DTT (5 mM), each of the four deoxyribonucleoside triphosphates (0.3 mM each), actinomycin D (10 μg/mL), and Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories; 20 units) was then added, and the reaction was incubated for 30 min at 45 °C. Control experiments demonstrated that the pyrimidine oligonucleotides used for triple-helix formation in this study did not inhibit these reverse transcription reactions. Primer extension products were collected by ethanol precipitation, suspended in formamide electrophoresis loading buffer, and resolved on 6% polyacrylamide–7.5 M urea sequencing gels in 0.5× TBE buffer (Sambrook et al., 1989). Dried gels were autoradiographed using Kodak XAR-5 X-ray film. Radioactive signals were subsequently quantitated by storage phosphor technology (Molecular Dynamics, Sunnyvale, CA). For each gel lane the signal corresponding to transcription initiation from the test promoter (S_t) was first normalized to the signal produced from the internal control promoter (S_c) to provide the ratio $R = (S_t)/(S_c)$. The corresponding transcription index is given by (R/R_-), where R_- is the value of R obtained in the absence of added oligonucleotide. Reported transcription indices are based on a minimum of two independent experiments.

Permuted DNA Fragment Mobility Analysis. Plasmid restriction fragments (200 ng) and radiolabeled oligonucleotide (1 μM) were combined in 10-μL binding reactions containing Tris-OAc, pH 6.8 (25 mM), NaCl (70 mM), $MgCl_2$ (20 mM), spermine tetrahydrochloride (0.4 mM), β-mercaptoethanol (10 mM), and bovine serum albumin (100 μg/mL). Oligonucleotide binding reactions were incubated for 30 min at 37 °C. Loading dyes [50% (v/v) glycerol, 0.05% (w/v) each xylene cyanol and bromophenol blue; 2 μL] were added, and the samples were loaded onto a 5% polyacrylamide [1:29 acrylamide/*N,N'*-methylenebis(acrylamide)] gel that had been preequilibrated at 4 °C in 1× TAM buffer [90 mM Tris-OAc, pH 6.0, 1 mM $Mg(OAc)_2$] at 10 V/cm with buffer recirculation. Electrophoresis was performed for 16 h. The gel was then stained with ethidium bromide, photographed under ultraviolet transillumination, and autoradiographed. Fragment mobility was measured relative to the absolute mobility (distance from gel origin) of the corresponding permuted fragment with the minimum value of d/l (defined in the legend to Figure 8A) as measured in the absence of specific oligonucleotide.

Results

Experimental Strategy. Effects of triple-helical DNA complexes on promoter function were determined by insertion of homopurine sequences and Sp1 binding sites in various configurations upstream of the TATA element in recombinant eukaryotic promoters. The structure of one such triple-helical complex is shown schematically in Figure 1. In this example, the 21 bp homopurine sequence has been designed to overlap 4 bp of a 10 bp consensus Sp1 binding site (Maher et al., 1989) inserted upstream of the TATA element of the adenovirus *E4* gene. Effects of triple-helical complexes on promoter function were measured in a soluble transcription extract prepared from cultured *Drosophila* cells (Parker & Topol, 1984). This experimental system is naturally devoid of Sp1 activity, permits facile adjustment of reaction conditions (e.g., pH), and presents no membrane barriers to oligonucleotide uptake (Courey & Tjian, 1988). Effects on transcription from modified *E4* templates were measured relative to an internal control promoter lacking both homopurine sequence inserts and Sp1 binding sites. A plasmid carrying basal promoter elements from the *fushi tarazu* (*ftz*) gene was used for this purpose. This experimental protocol is shown in Figure 2. The test plasmid (based on the *E4* gene) and the internal control plasmid (based on the *ftz* gene) were mixed and incubated for variable times with specific or nonspecific oligonucleotides (2.5 μM concentrations of compounds **1** or **3**, respectively) under conditions that favor triple-helix formation. This oligo-

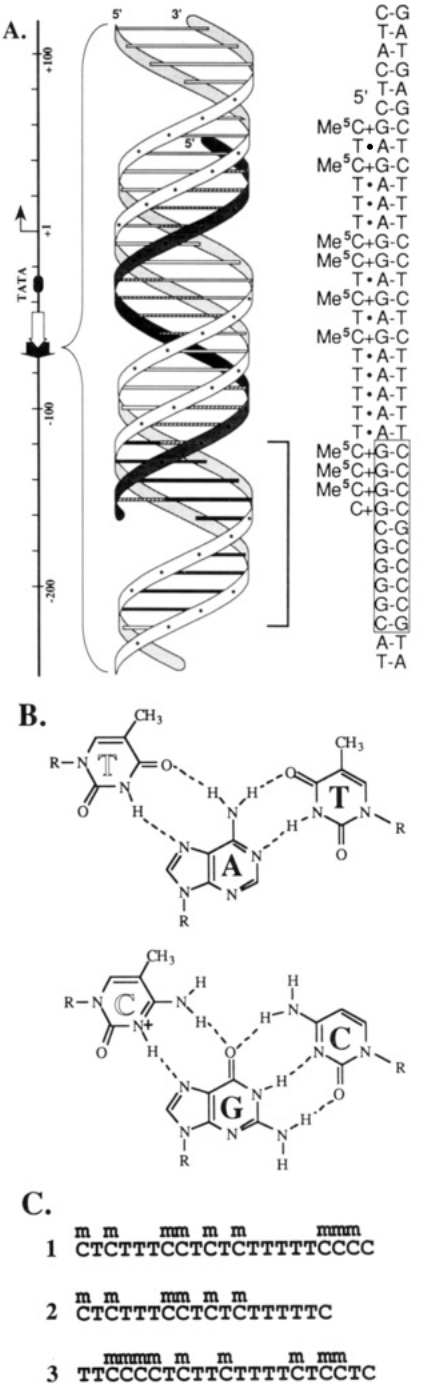

FIGURE 1: (A) Schematic representation of a triple-helical complex involving oligonucleotide **1** and template E4-2. An adenovirus *E4* promoter containing a TATA element and initiator has been modified by insertion of a 21 bp homopurine element (open arrow) overlapping a single Sp1 transcription factor binding site (filled arrow, left; bracketed base pairs, center; boxed sequence, right). Arrow orientation indicates 5′ to 3′ polarity of the purine-rich strands of the homopurine and Sp1 elements. (–) indicates Watson–Crick base pairing (right), whereas (·) and (+) indicate Hoogsteen hydrogen bonding between adenine and thymine, or guanine and protonated 5-methylcytosine, respectively, as shown in (B). (C) Nucleotide sequence of synthetic oligodeoxyribonucleotides used in this study. Oligomers **1** and **2** bind specifically to the homopurine element. Oligomer **3** is a control compound with the same base composition as **1**, but with a random base sequence.

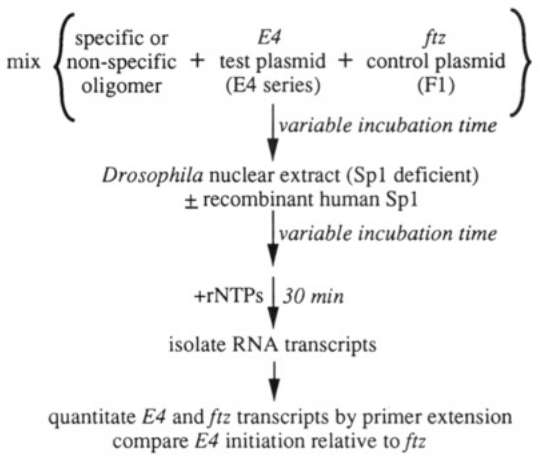

FIGURE 2: Protocol for in vitro transcription experiments. Oligonucleotides and templates (both test plasmid and internal control plasmid) were added, with or without a prebinding incubation, to *Drosophila* nuclear extract in the presence or absence of recombinant human Sp1-516C. Transcription reactions were incubated 30 min after initiation by addition of ribonucleoside triphosphates. RNA transcripts were subsequently isolated and quantitated as described under Experimental Procedures.

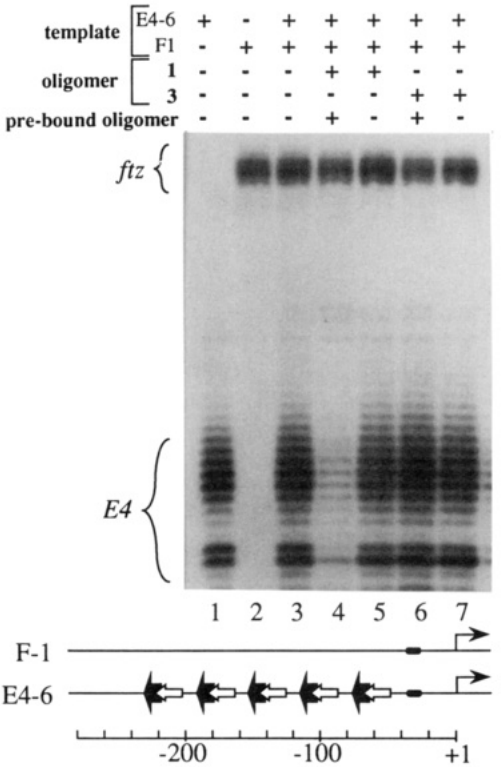

FIGURE 3: Autoradiogram of reverse transcripts obtained after transcription of templates E4-6 and F1 at pH 7.0 in the presence or absence of oligomer **1** or **3**, resolved by denaturing polyacrylamide gel electrophoresis. Homopurine elements (21 bp) are indicated by open arrows. Sp1 binding sites are indicated by filled arrows. In both cases, arrow direction indicates the 5′ to 3′ polarity of the purine-rich strand.

nucleotide concentration is 250-fold higher than the observed dissociation constant for the oligomer **1** complex at this homopurine sequence under similar conditions and should promote rapid and quantitative complex formation during a 30-min binding reaction (Maher et al., 1990). The templates were then incubated with *Drosophila* nuclear extract in the presence or absence of added recombinant Sp1-516C protein (1 μM final oligonucleotide concentration). Ribonucleoside triphosphates were typically added immediately to initiate transcription, but were withheld for various times in some experiments, as indicated. After a 30-min incubation in the presence of ribonucleoside triphosphates, RNA transcripts were isolated and quantitated by primer extension, electrophoretic

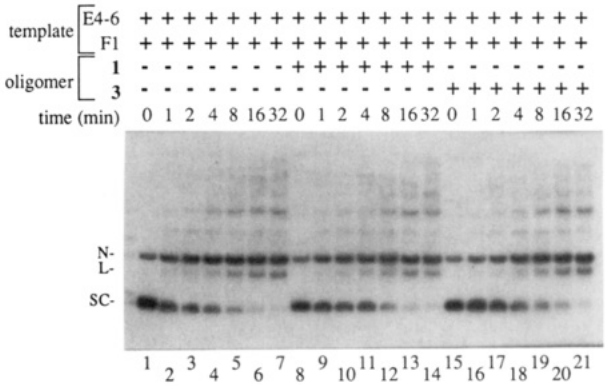

FIGURE 4: Time course of template fate. At various times after initiation of in vitro transcription of templates E4-6 and F1 (pH 7.0) in the presence or absence of oligomer **1** or **3**, template DNA was purified and resolved by agarose gel electrophoresis. The DNA was denatured, blotted to nylon membrane, and hybridized with radiolabeled probe specific for the E4-6 template (bottom strand of synthetic 40-mer duplex; see Experimental Procedures) according to standard procedures (Sambrook et al., 1989). Mobilities of supercoiled (SC), nicked open circular (N), and linear (L) plasmid DNA forms are indicated at left.

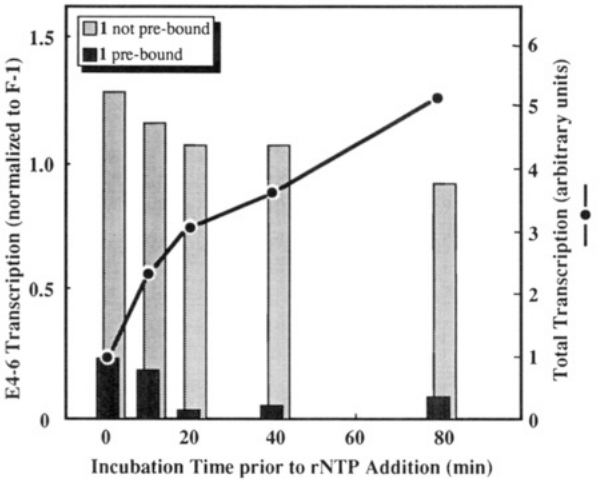

FIGURE 5: Time course of triple-helix-mediated repression. Transcription reactions with oligomer **1** either prebound (dark shading) or not prebound (light shading) to template E4-6 were assembled in the absence of ribonucleoside triphosphates. At various times after assembly (*x*-axis), 30-min transcription reactions (pH 7.5) were initiated by addition of ribonucleoside triphosphates. Bar heights (*y*-axis, left) reflect *E4* transcription, normalized to the internal F1 control template, and scaled relative to a value of 1.0 defined for each time point as the level of transcription from template E4-6 in the presence of control oligomer **3**. The effect of incubation time prior to ribonucleoside triphosphate addition on total transcription during the 30-min reaction (●) is also indicated (*y*-axis, right).

resolution of the extension products, and radiometric analysis.

Effects on Basal Transcription. Figure 3 shows the result of an experiment specifically testing effects of triple helices on basal (Sp1-independent) transcription using template E4-6, bearing five tandem copies of the homopurine/Sp1 element (Figure 1A). In this case, transcription reactions were performed at pH 7.0, in the absence of added Sp1. As shown in lanes 1 and 2 of Figure 3, primer extension products from the *E4* and *ftz* transcripts are readily distinguishable. *E4* transcripts are typically rather heterogeneous with respect to initiation site, as compared to transcripts originating from the *ftz* template. Interestingly, lanes 3 and 4 of Figure 3 show that triple helices formed by the binding of oligomer **1** repressed basal transcription from template E4-6 between 5- and 10-fold in a promoter-specific manner. Transcription after template incubation in the presence of nonspecific oligomer **3** (Figure 3, lane 6) was unaffected. Thus, an array of five triple-helical complexes upstream of the *E4* TATA element substantially repressed basal transcription in the absence of Sp1.

Repression Specificity and Template Fate. Specificity of triple-helix-mediated repression was examined according to several criteria. In all experiments oligomer **1** (specific sequence) and oligomer **3** (random sequence control with the same base content as oligomer **1**) were compared and oligomer **3** was found to have no effect on transcription. Therefore, repression was oligonucleotide-specific. Furthermore, promoter specificity was monitored in two ways. First, transcription quantitation in all experiments relied on normalization of the absolute level of transcription from the E4-series test promoter to the internal control signal provided by the F-series template. Second, linearized or supercoiled forms of recombinant template E4-6/F-1 (bearing convergent E4-6 and F1 promoters in cis, separated by 850 bp) were tested. These experiments gave results identical to those obtained with unlinked promoters, demonstrating that repression is specific to the E4-6 promoter even when the F1 control promoter is present on the same plasmid (data not shown). This result also indicates that triple-helix-mediated repression is not dependent on template superhelicity.

To eliminate the possibility that apparent repression was the result of a triple-helix-activated template degradation activity, E4-6 plasmid DNA was analyzed after extraction from standard transcription reactions at various times (Figure 4). Blot analysis indicated that supercoiled templates are gradually nicked and partially linearized during the course of 30-min transcription reactions (lanes 1–7). This pattern was unaltered after template preincubation and transcription in the presence of 1 μM concentrations of either specific oligomer **1** (lanes 8–14) or control oligomer **3** (lanes 15–21). Thus, the observed 5- to 10-fold repression of transcription from template E4-6 cannot be attributed to differential template cleavage or degradation in the nuclear extract.

Requirement for Triple-Helix Preassembly. It was observed that oligonucleotide-mediated repression required template preincubation with oligonucleotide, prior to addition of nuclear extract (Figure 3, lanes 4 and 5). This observation was investigated to determine if prolonged incubation of oligonucleotides and templates in the presence of nuclear extract would ultimately result in repression. Templates (with or without prebound triple-helical complexes) were incubated for various times with nuclear extract in the absence of ribonucleoside triphosphates (Figure 5). The pH of the nuclear extract was held at 7.5 in this experiment (nuclear extract activity was not strongly pH-dependent over the range 7.0–7.5). One set of templates was preincubated with oligomer **1** at ambient temperature, while the other was exposed to oligomer **1** only in the presence of nuclear extract. Ribonucleoside triphosphates were added at various times to initiate 30-min transcription reactions. As indicated by the right axis of Figure 5, it was observed that the overall level of transcription (E4-6 and F1 templates in the absense of oligomer) increased up to 5-fold with increasing template incubation time (up to 80 min) prior to ribonucleoside triphosphate addition. Templates preincubated with oligomer **1** did not become derepressed over this time period. Thus, triple-helix-mediated template repression at the time of nuclear extract addition appears to be stable for at least 80 min at pH 7.5. Furthermore, templates that had not been preincubated with oligomer **1** were not repressed upon subsequent incubation in the presence of nuclear extract. Similar results were obtained with

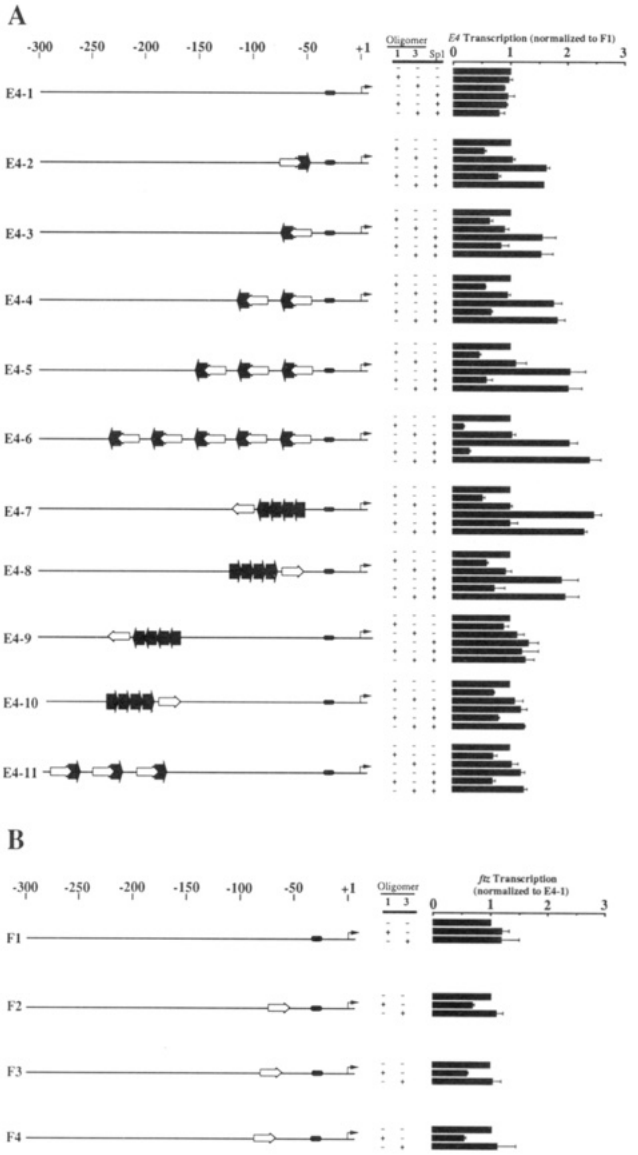

FIGURE 6: Repression by triple-helical DNA complexes. (A) Transcription initiation from E4-series templates was assayed at pH 7.0 by transcription of templates in the presence or absence of Sp1-516C after a prebinding step in the presence or absence of oligonucleotide **1** or **3**. Homopurine elements (21 bp) are indicated by open arrows. Sp1 binding sites are indicated by filled arrows. In both cases, arrow direction indicates the 5′ to 3′ polarity of the purine-rich strand. Bars at right indicate *E4* transcription, normalized to the internal control template and scaled relative to a value of 1.0 for *E4* transcription in the absence of both added oligonucleotide and Sp1 as described under Experimental Procedures. Error bars display standard error of the mean (SEM) derived from 2–5 independent replications. (B) Transcription initiation from F-series templates using E4-1 as internal control.

nuclear extract preparations at pH 7.0 (data not shown). Previous studies have shown that oligonucleotide association rate is not strongly pH-dependent, while dissociation rate is increased upon increasing pH (Maher et al., 1990). Under the present experimental conditions these results suggest that substantial triple-helix formation should occur within 30 min. Thus, activities present in the nuclear extract appear to alter these kinetics, possibly by decreasing either free oligomer concentration and/or double-helical DNA accessibility.

Effects on Sp1-Dependent Transcription Activation. We next wished to test whether Sp1 would derepress promoters bearing triple-helical complexes. It was therefore necessary to determine the dependence of triple-helix-mediated repression on the number, position, and distribution of triple-helical

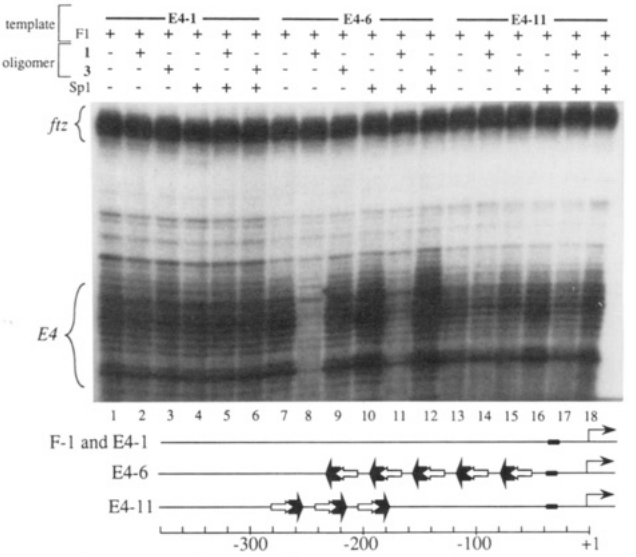

FIGURE 7: Autoradiogram of reverse transcripts obtained after transcription of templates E4-1, E4-6, and E4-11 (with template F1 as internal control) at pH 7.0 in the presence or absence of oligomer **1** or **3**, and in the presence or absence of Sp1-516C. Homopurine elements (21 bp) are indicated by open arrows. Sp1 binding sites are indicated by filled arrows. In both cases, arrow direction indicates the 5′ to 3′ polarity of the purine-rich strand. See Figure 6 for scale.

complexes and Sp1 sites in the test promoter. These results are shown in Figures 6 and 7. Transcription from promoters lacking homopurine or Sp1 elements was unaffected by the presence of oligomer or recombinant Sp1 (Figure 6A, template E4-1; Figure 7, lanes 1–6). In contrast, promoters bearing one, two, or three copies of the homopurine/Sp1 element shown in Figure 1 were repressed after preincubation in the presence of oligomer **1** and were activated by recombinant Sp1 in the absence of oligomer **1** (Figure 6A, templates E4-2, E4-3, E4-4, and E4-5). Promoters bearing single triple-helical complexes spaced 26 or 13 bp (in opposite orientations) from the TATA element (templates E4-2 and E4-3) gave similar transcription results. Basal transcription from these templates was inhibited (±SEM) to 0.54 ± 0.03 to 0.64 ± 0.05 of control levels. Addition of Sp1 activated transcription from both promoters by approximately 1.5-fold in the absence of oligomer **1**, but gave little activation when triple-helical complexes were present. Templates E4-4 and E4-5 contain two or three copies of the homopurine/Sp1 element, and triple-helical complexes at these sites repressed transcription to 0.56 ± 0.01 and 0.46 ± 0.03 of control values. Addition of Sp1 in the absence of oligomer **1** activated transcription to 1.76 ± 0.14 and 2.05 ± 0.27 of control values, respectively, but again failed to substantially derepress transcription in the presence of triple-helical complexes. The response of template E4-6 to oligomers has been described above. Addition of Sp1 in the absence of triple-helical complexes activated transcription from this template by more than 2-fold, but again was not observed to substantially relieve triple-helix-mediated repression (Figure 7, lanes 7–12).

The results described above suggest that triple-helical complexes repress basal transcription independent of their potential to occlude Sp1 binding. To further explore this observation, we measured transcription from promoters bearing triple-helical complexes that do not overlap Sp1 sites. Single homopurine elements flanked by four copies of high-affinity Sp1 sites from the SV40 early promoter were tested in either orientation 18 bp upstream from the *E4* TATA element (Figure 6A, templates E4-7 and E4-8). Transcription was again found to be relatively insensitive to insert orientation.

Oligomer **1** repressed basal transcription from templates E4-7 and E4-8 by approximately 2-fold. Observed template activation by Sp1 (2.47 ± 0.13 and 1.9 ± 0.3 of control values, respectively) in the absence of oligomer **1** was again largely eliminated in the presence of triple-helical complexes. When these inserts were moved further upstream (139 bp from the *E4* TATA element), both triple-helix-mediated repression and Sp1 activation were substantially diminished (Figure 6A, templates E4-9 and E4-10). A similar distance-dependent diminution of repression and activation was observed for three copies of the homopurine/Sp1 overlapping element when inserted 145 bp upstream from TATA (Figure 6A, template E4-11; Figure 7, lanes 13–18).

We wished to determine if triple-helix-mediated repression by single homopurine elements was somehow dependent on the presence of Sp1 binding sites, regardless of their occupancy by Sp1. Templates F2, F3, and F4 were constructed using the recombinant basal *ftz* promoter from F1 (Figure 6B). When transcribed in the presence of internal control template E4-1, F-series templates F2, F3, and F4 were repressed by oligomer **1** to 0.69 ± 0.03, 0.61 ± 0.02, and 0.54 ± 0.03 of controls, respectively. Thus, triple-helix-mediated repression can occur in the context of the *ftz* basal promoter, is independent of the presence of Sp1 binding sites, and is not strongly dependent on precise triple-helix position in the range 18–30 bp upsteam of the TATA element.

Position Dependence of Activating and Repressing Sequence Elements. Taken together, the data presented in Figure 6 provide evidence for general relationships between the distributions of activating and repressing elements and promoter function observed in these experiments. When analyzed independently, activation by recombinant Sp1 and repression by triple-helical complexes appear to depend upon the number of complexes of each type, inversely weighted by their distance from the TATA element.

Effects on DNA Flexibility. The data presented in Figure 6 suggest that repression by triple-helical complexes upstream of the TATA element occurs predominantly at the level of basal transcription. An obvious possibility is that repression is due to inhibition of proximal initiation complex assembly. While such inhibition could play a role for triple-helical complexes adjacent to TATA, this mechanism does not readily explain repression at greater distances, nor does it explain increased repression by additional triple-helical complexes positioned further upstream of TATA. We therefore considered alternative mechanisms involving the possibility that oligonucleotide-directed triple-helix formation bends or stiffens the target double helix in a manner that might be antagonistic to promoter function. Discrete alterations in DNA helix axis trajectory are predicted to occur at transitions between DNA helical forms differing in base pair tilt. If such anomalies are phased at odd multiples of half the helical repeat unit, the local bends are predicted to accumulate in a plane, resulting in a uniformly curved structure. Such anomalies in the structure of promoter DNA might inhibit proper assembly of the proximal initiation complex. Comparison of available structural models for double-helical and triple-helical DNA polymers suggests that base pair tilt may differ significantly between these helix families. Upon extrapolation to the case of oligonucleotide-directed triple-helix formation at homopurine target sites, reported values for the base pair tilt of B-form double-helical DNA (ca. −5°; Arnott & Selsing, 1972) and A′ (RNA-like) triple-helical DNA (ca. 8.5°; Arnott et al., 1976) suggest that conservation of base stacking at junctions between these helix forms could result in a deformation of the helix axis by as much as 13.5°. This junctional bending model predicts that, depending on the length and spacing of triple-helical complexes in a region of double-helical DNA, the polymer might adopt very different higher order structures ranging from multiply kinked to uniformly curved. Similar extrapolation of polymeric helical repeat parameters (10.5 bp/turn for double-helical DNA and 12 bp/turn for triple-helical DNA) to the case of oligonucleotide-directed DNA triple-helix formation suggests that coherent bending should be maximized at certain triple-helical complex distributions, e.g., 18 base triplets separated by 16 or 26 bp (Arnott et al., 1976; Arnott & Selsing, 1972).

An alternative stiffening model for the effect of triple-helix formation on double-helical DNA is based upon the reasonable prediction that three-stranded DNA helices may exhibit reduced flexiblity and correspondingly increased persistence lengths in solution relative to double helices. This prediction follows from consideration of the increased radial charge density in a triple helix relative to a double helix. In this stiffening model, small changes in triple-helical complex length and spacing in a region of double-helical DNA are not expected to substantially alter the overall trend toward loss of regional flexibility. Regions of increased DNA stiffness within a eukaryotic promoter might repress transcription by interfering with the ability of transcription factors to deform DNA into a functional proximal initiation complex.

An experimental approach for evaluating these models is based on their contrasting predictions concerning the unperturbed root-mean-square end-to-end distance $[(\langle r_0 \rangle)^{1/2}]$ of DNA fragments bearing such anomalies. In the junctional bending model, the case of triple-helical complexes arrayed to give a coherent bend should lead to a decrease in $(\langle r_0 \rangle)^{1/2}$. In the stiffening model, all arrangements of triple-helical complexes should act to increase the value of $(\langle r_0 \rangle)^{1/2}$. An indirect experimental method for measuring effects on $(\langle r_0 \rangle)^{1/2}$ in solution is provided by assays of permutation-dependent changes in the electrophoretic mobility of DNA fragments in polyacrylamide gels (Wu & Crothers, 1984). Such assays measure changes in the electrophoretic mobility of DNA fragments bearing inherent or ligand-induced structural perturbations as the position of the perturbation is changed within the DNA fragment. In the case of ligand-induced structural perturbations, this approach focuses not on mobility shifts due to ligand binding, but on mobility shifts due to changing the position of the bound ligand. Application of this assay is based on the prevailing view that shape selectivity between molecules of identical mass and electric charge reflects the facility with which molecules proceed through the polyacrylamide matrix in a wormlike manner. For molecules of identical mass and charge, increasing values of $(\langle r_0 \rangle)^{1/2}$ should increase electrophoretic mobility, while decreasing values of $(\langle r_0 \rangle)^{1/2}$ should have the opposite effect (Anderson, 1986). Permutation-dependent mobility assays exploit the expectation that any such mobility anomalies will be maximized when the bent or stiffened region is centrally rather than terminally positioned in a DNA fragment (Wu & Crothers, 1984).

Relative electrophoretic mobilities of circularly permuted DNA restriction fragments bearing various distributions of triple-helical complexes were determined using the approach diagrammed in Figure 8A. As a control, insert A contained phased tracts of A·T base pairs that cause a well-documented bend in kinetoplast DNA (Wu & Crothers, 1984). Insert B supports a single triple-helical complex involving oligomer **1** (21 base triplets). Inserts C and E support two and five complexes of this type, respectively, separated by 19 bp seg-

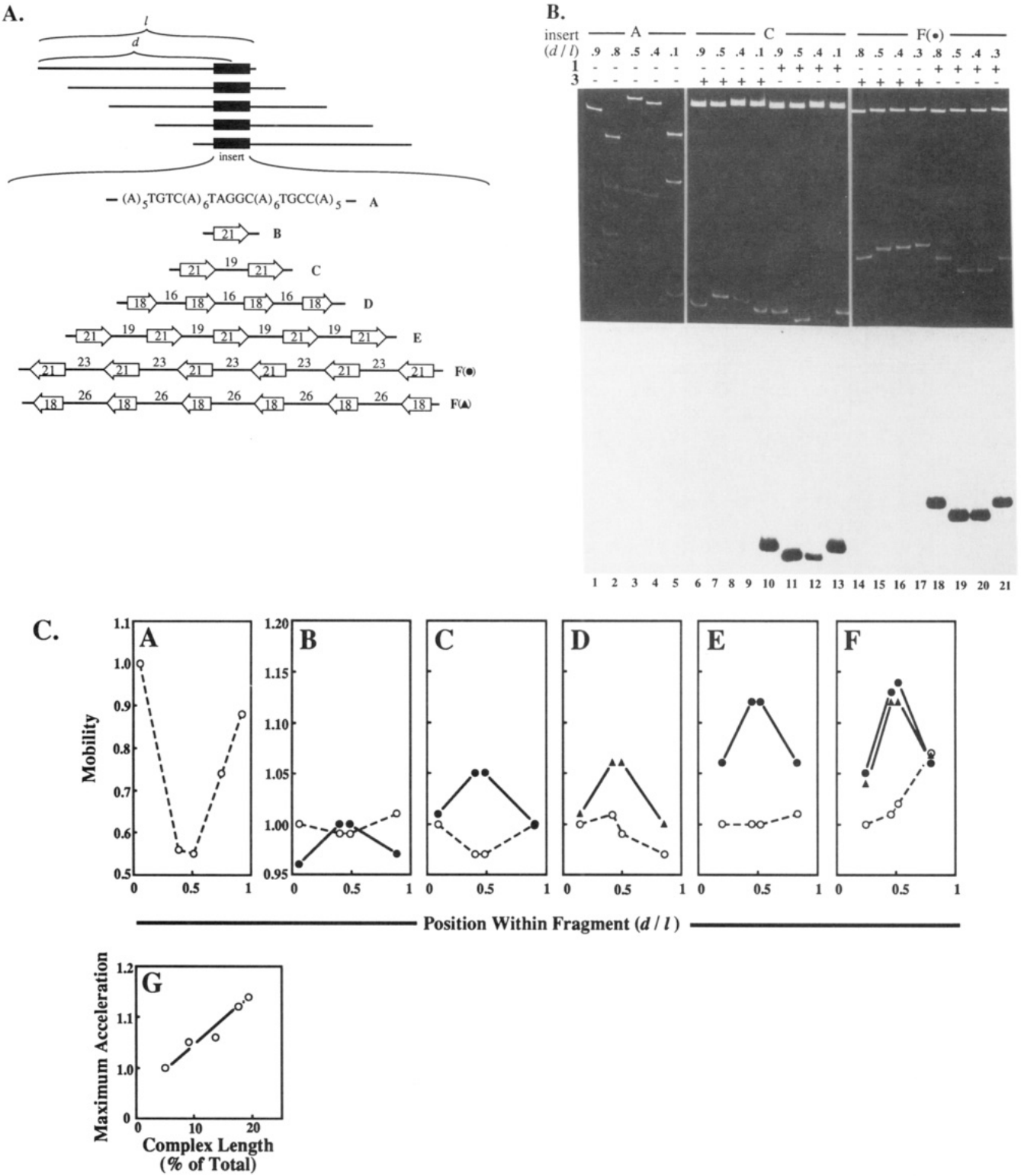

FIGURE 8: Permutation-dependent triple-helix-induced DNA mobility shifts. (A) Schematic diagram of permuted DNA restriction fragments carrying an insert that is either know to be inherently bent (insert A) or whose bending/stiffening character is unknown [inserts B–E, F(●), and F(▲)]. Values of d (the distance from the insert center to the left restriction fragment terminus) and l (the restriction fragment length) were used to define the value (d/l) specifying the insert position within the permutation series. (B) Ethidium bromide stained agarose gel (above) and autoradiogram (below) displaying permutation-dependent deceleration for bent control insert A (lanes 1–5; relevant fragment corresponds to highest mobility band in each lane) and permutation-dependent acceleration for triple-helical complexes involving inserts C and F(●) and radiolabeled oligomers **1** and **3** (lanes 6–13 and 14–21, respectively). (C) Quantitation of permutation-dependent DNA mobilities for the inserts tested in these experiments. Mobilities were assigned using the method described under Experimental Procedures. Panels A–F correspond to inserts A–F, where the mobilities of permuted fragments carrying these inserts in the presence of oligomers **1** (●), **2** (▲), or **3** (○) are indicated. No oligomer was present in the case of panel A. Panel G depicts the relationship between the total triple-helical character of the restriction fragment (% of restriction fragment length) and maximal mobility acceleration.

ments. Insert D supports four triple-helical complexes involving oligomer **2** (18 base triplets), separated by 16 bp spacer segments. Inserts F(●) and F(▲) both support six triple-helical complexes. In insert F(●), the complexes involve oligomer **1** (21 base triplets), separated by 23 bp segments. In insert F(▲), the complexes involve oligomer **2** (18 base triplets),

separated by 26 bp segments. A key design feature of these inserts is the length and phasing of triple-helical complexes. According to the junctional bending model, the helical repeat parameters described above suggest that inserts A, D, and F(▲) should be curved upon triple-helix formation, due to the accumulation of coherent junctional bends. Because it is predicted to decrease $(\langle r_0 \rangle)^{1/2}$, such anomalies are expected to *decrease* fragment mobility in a permutation-dependent manner, with the most extreme retardation occurring when the anomaly is centrally located. Upon triple-helix formation, this model predicts that junctional bends in inserts B, C, E, and F(●) should not be coherent, leading to mildly kinked, rather than curved, structures. Such anomalies are not expected to product substantial permutation-dependent electrophoretic retardation. In contrast, the stiffening model predicts that all inserts will cause stiffening upon triple-helix formation, with the magnitude of the effect proportional to the number of induced base triplets. As such stiff regions tend to increase the value of $(\langle r_0 \rangle)^{1/2}$ for fragments bearing them, *increased* electrophoretic mobility is predicted, with the most extreme enhancement occurring when the anomaly is centrally located.

These predictions were tested by electrophoresis of permuted restriction fragments bearing triple-helical complexes at pH 6.0 where complexes are sufficiently stable to remain intact throughout the course of the experiment. Permuted fragments were then visualized by staining with ethidium bromide. Results of experiments of this type are shown in Figure 8 (panels B and C). Under these experimental conditions, insert A severely retards fragment mobility in a permutation-dependent manner, as predicted for a curved anomaly (highest mobility fragments in Figure 8B, lanes 1–5, and Figure 8C, graph A). As shown in Figure 8 (panels B and C), in contrast to the predictions of the bending model, all DNA restriction fragments bearing triple-helical DNA complexes exhibited permutation-dependent acceleration rather than retardation. Furthermore, the degree of maximum mobility enhancement for each insert was roughly proportional to the total number of base triplets present upon triple-helix formation, regardless of the precise lengths and spacings of triple-helical complexes (Figure 8C, graph G). This observation confirms that oligonucleotide-directed triple-helix formation changes the physical properties of the target double helix in a manner consistent with DNA stiffening. These results do not support the junctional bending model.

Discussion

Triple-Helix Effects on Basal Transcription. We report that site-specific triple-helical DNA complexes upstream of the TATA element are promoter-specific repressors of eukaryotic transcription initiation by RNA polymerase II. It has previously been demonstrated that site-specific DNA triple-helix formation can block Sp1 binding (Maher et al., 1989). We extended this observation to a functional context in which Sp1 acts as an activator of transcription. We find that triple-helical complexes act predominantly as repressors of basal transcription and can do so at a modest distance, regardless of whether such complexes overlap Sp1 binding sites. Moreover, it was found that Sp1 is incapable of relieving triple-helix-mediated repression. Repression is strictly dependent on both oligonucleotide sequence and tight linkage of the homopurine target elements to the test promoter. Thus, under no circumstances was nonspecific oligomer **3** observed to affect transcription from any experimental or internal control template. Moreover, specificity of repression was documented through the use of internal control promoters and was observed on supercoiled and linearized templates, as well as in the case of internal control and experimental promoters contained within a single plasmid. Together with the demonstration that promoter-specific repression is not accompanied by increased template degradation, these observations indicate that repression reflects promoter-specific disruption of transcription initiation.

Triple-Helix Preassembly Requirement. We find that transcriptional repression absolutely requires the preassembly of triple-helical complexes before addition of *Drosophila* nuclear extract. This requirement cannot be overcome by prolonged template incubation in the presence of extract and DNA oligomer at pH 7.0 or 7.5 prior to addition of ribonucleoside triphosphates. Previous experiments conducted in the absence of nuclear extract have examined the rate and extent of triple-helix formation as a function of oligonucleotide concentration between pH 6.8 and 7.2 (Maher et al., 1990). On the basis of these results, the experimental oligonucleotide concentration (1 μM) should have been sufficient to permit substantial triple-helix formation on naked DNA within 20 min at pH 7.0. The observed preassembly requirement may therefore reflect one or more activities of the nuclear extract. The presence of high concentrations of DNA binding proteins in nuclear extract preparations may foster nonspecific interactions with template DNA, resulting in structures that inhibit oligonucleotide-directed triple-helix formation. Such nonspecific interactions may also be responsible for the observation that functional initiation complexes are assembled on only a small fraction of added templates upon incubation in nuclear extracts (Croston et al., 1991; Parker & Topol, 1984). Thus, the nuclear extract system may erect an unnatural barrier to triple-helix formation that would not exist in the context of nuclear chromatin where DNA:protein stoichiometry is much different. It is also possible that the nuclear extract contains sufficient single-stranded DNA binding or hydrolytic activities to reduce free oligomer concentration below that required to drive complex formation under the kinetic and thermodynamic constraints of this assay.

It is interesting to compare the observed oligonucleotide prebinding requirement reported here to repression of in vitro transcription by histone H1 (Croston et al., 1991). Like triple-helical DNA complexes, H1 acts to repress basal transcription, albeit in a sequence- and promoter-nonspecific manner. It may be noteworthy that H1-mediated repression also requires prebinding of H1 to template DNA. No repression is observed if transcription factors and activating proteins are allowed to bind prior to H1. It is unknown whether similar mechanisms are responsible for the observed oligonucleotide prebinding requirement reported here.

Position Dependence of Repressing and Activating Sequence Elements. A general result of these experiments is the observation that repression by triple-helical complexes tends to increase with the number and proximity of such complexes to the TATA element. An even stronger relationship of this type is observed for transcriptional activation by bound Sp1-516C molecules. Beyond these general observations, particular distributions and orderings of activating and repressing elements are relatively unimportant. For example, changing the distance of a single triple-helical complex over several helical turns of DNA upstream of TATA had relatively little affect on the degree of repression (e.g., templates E4-2, E4-3, E4-7, E4-8, F2, F3, F4). Furthermore, order independence was observed when triple-helical complexes were placed adjacent to groups of Sp1 sites. Thus, repression was comparable whether or not triple-helical complexes were positioned to

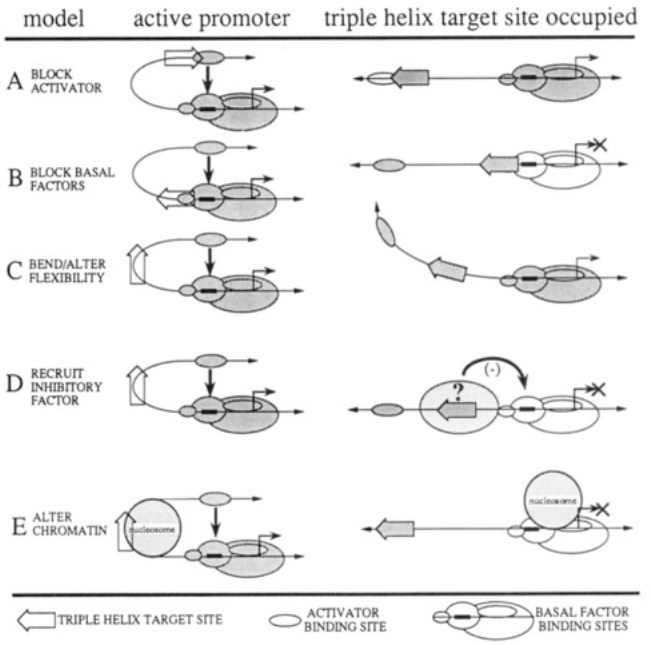

FIGURE 9: Five models suggesting possible mechanisms for repression of eukaryotic transcription initiation by triple-helical DNA complexes. See text for discussion.

intervene between the group of Sp1-516C monomers and the TATA element (templates E4-7, E4-8, E4-9, E4-10).

Comparison of Sequence-Specific and -Nonspecific Repressors. Our observations support the proposition that preassembled triple helices exert promoter-specific repression that cannot be substantially reversed in the presence of Sp1-516C. In this regard, the effects of triple-helical complexes may be compared with those observed for nonspecific repressors such as histone H1 (Croston et al., 1991). Transcription factors have been shown to enhance transcription in the presence of H1 through mechanisms involving either derepression alone or derepression coupled to frank activation (Croston et al., 1991). Interestingly, although it acts in the latter mode relative to repression by H1, Sp1 can neither derepress nor activate transcription in the presence of triple-helical complexes. In fact, triple-helical DNA complexes are sufficient to substantially block Sp1 activation even when the complexes do not overlap Sp1 binding sites (e.g., templates E4-7 and E4-8).

Models. We have considered possible mechanistic explanations for effects of triple-helical complexes on transcription initiation. Five plausible models for promoter-specific repression by triple-helical DNA complexes are shown in Figure 9.

Model A depicts inhibition of transcriptional activation, reflecting the original experimental design. Elimination of activator binding was expected to reduce transcription to the basal level observed in the absence of activator. As described above, this simple model cannot account for the observed ability of triple-helical complexes to exert substantial effects on the basal level of transcription, although it remains possible that inhibition of Sp1 binding is also occurring.

Model B suggests that promoter-specific repression by triple-helical complexes result from disruption of basal factor interactions at the promoter. Assembly of an RNA polymerase II transcription complex has been shown to protect a region extending from about 1.5 helical turns upstream of the TATA element to about 1.5 helical turns downstream from the initiation point (Buratowski et al., 1989). A subset of templates tested here contained triple-helical complexes that impinged on the 15 bp region upstream of TATA. However, several experimental results tend to argue against this model as the major mechanism for the observed results. For example, templates E4-2 and E4-3 are spaced 26 and 13 bp, respectively, from the TATA element, but are similarly repressed by triple-helix formation. Templates E4-3, E4-4, E4-5, and E4-6 all possess a homopurine sequence 13 bp upstream of the TATA element, but are subject to increasing repression in the presence on oligomer **1**. Furthermore, templates in the series F2, F3, and F4 show comparable repression by oligomer **1** in spite of the increasing separation of the proximal triple-helical complex from the TATA element (18, 24, and 30 bp, respectively). These results suggest that repression is not dictated solely by the most TATA-proximal complex, although direct effects on the promoter may contribute in some cases.

Model C proposes that triple-helical complexes induce changes in the physical properties of promoter DNA that tend to inhibit assembly of the promoter into a productive initiation complex. In particular, bending at duplex–triplex junctions and reduction of DNA flexibility were considered and explicitly tested. It was found that the electrophoretic mobility of DNA restriction fragments bearing triple-helical complexes depended on the position of the complexes within the fragment. We conclude that changes in mobility reflect changes in average molecular shape. Because the electrophoretic anomaly is opposite to that observed for curved DNA fragments, this result supports the proposition that short triple-helical complexes positioned near the center of a larger DNA molecule can act to increase its apparent persistence length. Similar interpretations have been proposed for electrophoretic anomalies of this type arising at certain (A/T)-rich DNA sequences known to resist nucleosome assembly or to be relatively rigid on the basis of other physical studies (Anderson, 1986).

How triple-helix formation substantially upstream of TATA might limit assembly of the basal (Sp1-independent) initiation complex is presently uncertain, but suggests the possibility of propagation or amplification of a primary local effect by other factors. Model D suggests that repression by triple-helical DNA structures may be mediated by an unknown activity in the nuclear extract that recognizes some aspect of the altered DNA structure and acts to repress transcription. The existence of triple-helix-specific binding activities is not implausible in light of evidence favoring a three-stranded structural model for H-DNA elements in vivo (Davis et al., 1989; Lee et al., 1987; Parniewski et al., 1990). Furthermore, although natural cytosine methylation is notably absent in *Drosophila*, the presence of DNA binding proteins with preference for 5-methylcytosine could contribute to the formation of a repressive nucleoprotein complex at the triple helix (Boyes & Bird, 1991). The experiments reported here do not support or refute this model, except that we have excluded the specific possibility that the postulated activity acts by nicking or cleaving double-helical DNA at sites of triple-helix formation.

Model E addresses the possibility that triple-helix formation could affect patterns of chromatin assembly on DNA and therefore activate or repress transcription. Evidence that chromatin assembly can act as a general inhibitor of transcription suggests that sequence-specific inhibition of nucleosome assembly could have effects on the accessibility of promoter DNA to basal transcription factors including RNA polymerase II (Croston et al., 1991; Weintraub, 1984). Although it is uncertain to what extent authentic chromatin assembly occurs in the *Drosophila* nuclear extract under our experimental conditions, this model suggests mechanisms that may apply to future studies of chromatin assembly after in-

duction of triple-helical structures by oligonucleotide binding or H-DNA strand rearrangement.

Comparison to Triple Helices of the Purine·Purine·Pyrimidine (Purine Motif) Type. It is relevant to compare the results reported here to those described by Hogan and coworkers for triple-helix formation at a purine-rich element upstream of the human c-*myc* gene by a purine-rich oligomer (Cooney et al., 1988). These workers documented oligonucleotide-specific promoter repression consistent with that reported here, without addressing the issue of promoter specificity. It is also unclear to what extent the observed requirement for triple-helix preassembly reported here differs from this previous report. Purine motif triple-helical complexes have subsequently been shown to involve purine oligonucleotide binding *antiparallel* to the purine strand of the target double helix (Beal & Dervan, 1991). Triple-helix formation in the purine motif has the advantage of pH independence. However, perhaps because they are not profoundly G-rich, homopurine target sites chosen for the current study assemble into less stable triple-helical structures in the purine motif than in the pyrimidine motif (Hacia, Wold, and Dervan, unpublished observations). Where tested, triple-helical complexes formed using the purine triple-helix motif gave qualitatively similar (although quantitatively diminished) repression relative to that reported here for the pyrimidine triple-helix motif (data not shown).

Extension to Transcription Elongation and Termination. The experiments reported here specifically address attempts to artificially regulate transcription initiation by oligonucleotide-directed DNA triple-helix formation. It will be important to extend these results to transcriptional elongation and termination by RNA polymerase II. Of interest in this regard are preliminary studies indicating that noncovalent triple-helical DNA complexes do not inhibit transcriptional elongation by bacteriophage RNA polymerases (Maher, Povsic, Dervan, and Wold, unpublished observations).

Conclusions. We demonstrate that noncovalent but site-specific oligonucleotide interactions with double-helical DNA can serve to repress transcription initiation in a suitably designed in vitro system. If the observed gene-specific repression by triple-helical complexes extends to nuclei of living cells, our results suggest an unanticipated flexibility in the precise location of triple-helical complexes relative to transcription factor binding sites. Such flexibility would not have been expected on the basis of a simple factor occlusion model. Moreover, unlike the nuclear extract system, enhancement of transcription from great distances is common in cells. In such cases it may be possible to more definitively separate triple-helix effects on upstream activators from effects on basal transcription. Thus, factor occlusion or local DNA stiffening due to triple-helix formation might uncouple enhancer–promoter interactions. Finally, the postulated inhibition of basal promoter activity at modest distances from the site of transcription initiation suggests that naturally occurring potential triple-helix target sites, which do not generally overlap known protein binding sites, might nonetheless have functional significance.

ACKNOWLEDGMENTS

We gratefully acknowledge C. Parker and M. Muhich for plasmids pG_5E4T and pF1, and for advice and technical assistance in nuclear extract preparation. Plasmid pHW122 was the generous gift of H.-M. Wu. We are pleased to acknowledge G. Schroth for providing plasmid pCY7 and for helpful discussions and D. Crothers for discussion and encouragement. *E. coli* strain C600 Δ*lon* and plasmid pSp1-516C were the generous gifts of R. Johnson and R. Tjian, respectively. We thank S. Tavtigian, S. Strobel, S. Singleton, and J. Hacia as well as other members of the Dervan and Wold laboratories for technical assistance and discussion.

Registry No. 40-mer duplex, 137593-83-0; 73-mer duplex, 137593-82-9; 26 bp duplex, 137593-81-8; 44-mer duplex, 137593-84-1.

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Structural Organization and Regulatory Regions of the Human Medium-Chain Acyl-CoA Dehydrogenase Gene[†,‡]

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Received July 16, 1991; Revised Manuscript Received September 24, 1991

ABSTRACT: Medium-chain acyl-CoA dehydrogenase (MCAD) is a highly regulated mitochondrial flavoenzyme that catalyzes the initial reaction in fatty acid β-oxidation. Deficiency of MCAD is a common inherited defect in energy metabolism. We have previously shown that the mRNA encoding MCAD in an MCAD-deficient child is homozygous for the point mutation A^{985} to G [Kelly et al. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9236-9420]. To define the molecular basis of MCAD deficiency and as an initial step in the study of the regulation of MCAD gene expression, we determined the structure and organization of the human MCAD gene. The gene is comprised of 12 exons which span 44 kb of DNA. Comparison of the MCAD gene to MCAD mRNAs from the MCAD-deficient child revealed that missplicing was common, resulting in a variety of exon deletions and intron insertions. The MCAD gene promoter region is extremely GC-rich and lacks prototypical TATA and CAAT boxes. Several regions upstream of the promoter are homologous with mitochondrial enhancers purportedly involved in coordinate expression of nuclear genes encoding mitochondrial proteins. Transfection of chimeric plasmid constructs with 299 bp of upstream sequence into HepG2 cells revealed high-level transcriptional activity. We conclude that the precursor MCAD mRNA is misspliced to a high degree and complexity in association with the G^{985} mutation and the MCAD gene contains a strong promoter which shares some structural features with other "housekeeping" genes encoding mitochondrial proteins.

Medium-chain acyl-CoA dehydrogenase (MCAD; 2,3-oxidoreductase, EC 1.3.99.3)[1] is a mitochondrial matrix flavoprotein which catalyzes the first reaction in the β-oxidation of straight-chain fatty acids (Beinert, 1963). It forms an enzyme family with short- and long-chain acyl-CoA dehydrogenases. These three enzymes are homotetramers with subunits of similar size, but they are immunologically distinct with different amino acid sequences (Ikeda et al., 1985). MCAD requires medium-chain-length acyl-coenzyme A substrates, contains FAD, and ultimately transfers electrons to electron-transfer flavoprotein. Like most mitochondrial proteins, MCAD is encoded by a nuclear gene, is synthesized in the cytosol as a larger precursor with an NH_2-terminal transit peptide, and is subsequently imported into mitochondria with proteolytic processing to the mature form (Warren, 1987; Kelly et al., 1987).

Inherited deficiency of human MCAD was first described in 1983 and is now recognized as a common inherited metabolic disorder (Kolvraa et al., 1983; Rhead et al., 1983; Stanley et al., 1983). Clinical manifestations of MCAD deficiency include fasting hypoglycemia, recurrent Reye-like syndrome, or sudden death during the first 2 years of life. MCAD-deficient individuals may also be completely asymptomatic (Bougneres et al., 1985; Treem et al., 1986; Duran et al., 1986). If fasting crises are avoided, MCAD-deficient individuals develop normally.

As an initial step in the molecular analysis of MCAD deficiency and to study the regulation of expression of MCAD, we previously isolated and characterized cDNA clones encoding human and rat MCAD mRNAs (Kelly et al., 1987,

[†] This work was supported by Grants AM 20407 (A.W.S. and Z.Z.) and GM 29076 (J.-J.K.) from the National Institutes of Health. A.J.W. is a Fellow of the American Heart Association, Missouri Affiliate. D.P.K. is the recipient of a Lucille P. Markey Scholar Award.

[‡] The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J05355.

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[1] Abbreviations: MCAD, medium-chain acyl-CoA dehydrogenase; bp, base pair(s); PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; UTR, untranslated region.

0006-2960/92/0431-81$03.00/0