growth of microtubules (Wilson et al., 1976, 1982; Na & Timasheff, 1982a). If this is correct, the vinblastine inhibition of microtubule assembly must be linked to the same vinblastine binding site on the protein as the self-association reaction. This mechanism is rendered even more plausible now in view of the strong enhancement by magnesium ions of the vinblastine-induced tubulin self-association reported here.

Registry No. Mg, 7439-95-4; $MgCl_2$, 7786-30-3; vinblastine, 865-21-4.

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Nucleotide Sequence and Nuclease Hypersensitivity of the Chinese Hamster Dihydrofolate Reductase Gene Promoter Region†

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ABSTRACT: We have sequenced the 1240 base pairs (bp) upstream from the translation start site of the hamster dihydrofolate reductase (DHFR) gene. The DNA in the 5′ flanking region contains several elements that are homologous in both sequence and relative location to corresponding elements in the human and murine DHFR genes: an 11-bp element adjacent to the ATG codon, a 19-bp element that coincides with the major transcription start site, and two 29-bp upstream elements that are represented 4 times in the murine DHFR gene but only once in the human gene. Two clusters of short, G/C-rich elements conforming to the consensus binding sequence for the transcription factor Sp1 are located in the upstream region in all three genes. The symmetrical placement of the G/C boxes coincides with a symmetrical DNase I hypersensitive pattern in the chromatin, suggesting that the Sp1 protein may be involved in maintaining chromatin structure in this region.

Dihydrofolate reductase (DHFR)[1] catalyzes the reduction of folate to dihydrofolate and, subsequently, to tetrahydrofolate. Reduced folates are required in the biosynthesis of purine nucleotides, thymidine, and glycine. The gene encoding DHFR is expressed in all tissues, and its product has therefore been termed a "housekeeping" enzyme.

Several lines of evidence suggest that the transcription of

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[1] Abbreviations: DHFR, dihydrofolate reductase; MTX, methotrexate; EDTA, ethylenediaminetetraacetic acid.

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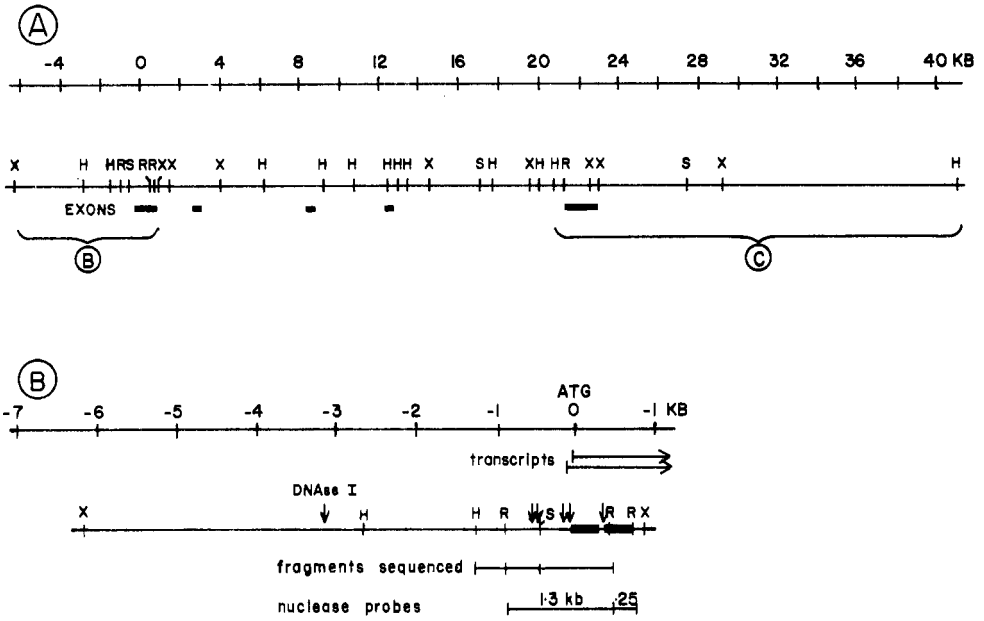

FIGURE 1: Map of the Chinese hamster DHFR locus. In panels A and B, the scale is adjusted so that the A in the ATG codon is +1 bp. This convention is observed throughout the text. (A) Extended map of the DHFR locus, showing the placement of exons within the gene (black boxes, 8), and the locations of the 5′ *Xba*I fragment (B) and the 3′ *Hin*dIII fragment (C) that were analyzed for the presence of nuclease hypersensitive sites in chromatin. For simplicity, *Eco*RI sites are shown only in *Xba*I fragment B and in the left end of *Hin*dIII fragment C. The latter *Eco*RI site defines a 500- bp *Hin*dIII/*Eco*RI probe that was used in nuclease studies on chromatin (see Results). (B) Detailed map of the 7.2-kb *Xba*I fragment that spans the 5′ end of the DHFR gene. DHFR exons are indicated with black boxes. The positions of the major and minor transcription start sites at −63 and −107, respectively, are shown (Mitchell et al., 1986), as well as the fragments that were subcloned into M13 for sequencing studies. The positions of the 1.3-kb and 250-bp *Eco*RI fragments that were utilized as probes to map the nuclease hypersensitive sites in this *Xba*I fragment are also indicated. The approximate positions of the DNase I hypersensitive zones are indicated by vertical arrows.

the DHFR gene, as well as subsequent processing of its mRNA, may be regulated during the cell cycle and in response to environmental changes. In methotrexate-resistant murine fibroblasts, the rate of transcription of the DHFR gene increases after serum stimulation of quiescent cells (Hendrickson et al., 1980; Santiago et al., 1983) and in cells entering the S period after synchronization by mitotic selection (Farnham & Schimke, 1985). In another methotrexate-resistant murine cell line, no increase in DHFR transcription was detected after replating growth-arrested cells at low density; however, an increase in the half-life of DHFR mRNA was observed, as well as an increase in the rate of processing of nuclear transcripts (Leys & Kellems, 1981). Additional studies with transfected DHFR minigenes suggest that elements mapping in the 3′ untranslated region of the gene may be responsible, in part, for the regulation of expression during the cell cycle (Kaufman & Sharp, 1983). At present, however, the molecular basis for alterations in expression of the DHFR gene are not well understood.

The isolation of recombinant clones representing the murine, hamster, and human DHFR genes has been facilitated by screening genomic DNA libraries from methotrexate-resistant cell lines in which the gene is amplified to high copy number (Nunberg et al., 1980; Carothers et al., 1983; Milbrandt et al., 1983; Chen et al., 1984). The exon/intron arrangements of each of the three genes have been determined, and the 5′ flanking regions and the exons of the human and murine genes have been sequenced (Chen et al., 1984; McGrogan et al., 1985). In addition, the chromatin in the 5′ flanking regions of the hamster and human DHFR genes has recently been shown to be hypersensitive to several nucleases (Mitchell et al., 1986; Shimada et al., 1986), indicating that the regulatory regions of these genes have the open chromatin configuration characteristic of other transcriptionally active genes [see Elgin (1981) for a review].

We have begun to study the regulation of the DHFR gene in a methotrexate-resistant Chinese hamster cell line, CHOC 400. In the present paper, we report on the primary sequence and chromatin configuration of the 5′ regulatory region of a gene that was cloned from CHOC 400 cells (Milbrandt et al., 1983). These studies illuminate striking similarities in the organization of 5′ flanking sequences in the Chinese hamster, murine, and human DHFR genes. In addition, the chromatin configuration in this conserved region appears to be similar in both hamster and human DHFR genes, as assessed by nuclease sensitivity measurements on isolated nuclei. These comparisons suggest certain sequence elements that are likely to be important for the transcription and/or regulation of the DHFR gene.

Materials and Methods

DNA Sequence Analysis. The DNA sequence of the 5′ flanking region of the DHFR gene was determined by the dideoxy chain termination method (Sanger et al., 1977), except that reactions were carried out at 37 °C and the NaCl concentration was 10 mM in the annealing and synthesis steps. Fragments for sequencing were subcloned into the M13 vectors, mp18 and mp19 (Yanisch-Perron et al., 1985), and were obtained from a 7.8-kb *Hin*dIII fragment that begins 1240 bp upstream from the ATG codon of the gene and extends into the middle of the third intron (see map, Figure 1A). The 350-bp *Hin*dIII/*Eco*RI fragment and the 420-bp *Eco*RI/*Sma*I fragment in this region were sequenced from both ends, utilizing the M13 17-bp universal primer. In addition, oligonucleotides representing the coding and noncoding strands were synthesized from the −690 to −670 region on the basis of the sequence determined above and were used as primers to confirm the sequence. Thus, the sequence from −1240 to −471 was determined for both strands. The 890-bp *Sma*I/*Eco*RI fragment that spans the −471 to +419 region was sequenced

from −471 to +20 by the universal primer and oligonucleotides representing both strands of the −310 to −290 region. In addition, an oligonucleotide representing the +1 to +20 coding sequence was synthesized on the basis of sequencing data provided by L. Chasin and co-workers (Mitchell et al., 1986). This primer was used to confirm the sequence in the −250 to +20 region on the noncoding strand. The subcloned fragments used for sequencing are shown in Figure 1B. Dideoxynucleotides were obtained from P-L Biochemicals, and Klenow fragment, vectors, and primer, from New England Biolabs.

Direct and inverted repeat sequences were identified by using the SEQH program (Goad & Kanehisa, 1982), and dot homology plots were constructed with the aid of the DIAGON program (Staden, 1982). Sequences of the 5′ flanking regions of the human and murine genes were taken from McGrogan et al. (1985) and Chen et al. (1984), respectively.

Cells and Tissue Culture. The cell line CHOC 400 is a Chinese hamster ovary derivative that is resistant to 800 μM methotrexate. CHOC 400 contains approximately 1000 copies of the DHFR gene, which are located predominantly in a single, large homogeneously staining chromosomal region (Milbrandt et al., 1981). Cell cultures were maintained as monolayers in a humidified atmosphere of 5% CO_2. The medium was Eagle's minimal essential medium (Earle's salts) supplemented with nonessential amino acids, gentamycin, 800 μM methotrexate, and 10% donor calf serum. Starvation medium lacking isoleucine was reconstituted from a Selectamine kit (GIBCO) and contained 10% dialyzed donor calf serum. In synchrony experiments, cultures were starved for isoleucine for 45 h in order to induce G1 arrest and were then transferred to complete medium containing 10 μg of aphidicolin/mL for 13 h. This protocol collects >95% of cells at the G1/S boundary (Heintz & Hamlin, 1982). All tissue culture supplies were obtained from GIBCO. Aphidicolin was supplied by the Drug Development Branch of the NIH.

Nuclease Studies. Cells in midexponential growth or at the G1/S boundary were removed from 15-cm tissue culture dishes by trypsinization and were washed twice with cold phosphate-buffered saline containing 2 mM calcium and 2 mM magnesium. After centrifugation in the cold at 800*g* for 3 min, the cell pellet from two plates was suspended in 4 mL of homogenization buffer (0.3 M sucrose/2 mM magnesium acetate/3 mM $CaCl_2$/10 mM Tris, pH 8.0/0.2% NP40/2 mM 2-mercaptoethanol/1 mM PMSF) at a concentration of 2×10^7 cells/mL. The suspension was dispersed with 20 strokes of a tight-fitting Dounce homogenizer and was layered onto 10 mL of pad buffer (2 M sucrose/5 mM magnesium acetate, 10 mM Tris, pH 8/2 mM 2-mercaptoethanol/1 mM PMSF) in a 15-mL Corex tube. For G1/S phase nuclei, the sucrose in the pad buffer was reduced to 1.5 M. Nuclei were pelleted in an HB-4 rotor (Sorvall) at 8000 rpm for 30 min at 0 °C, the supernatant fluid was aspirated, and the pellets were drained and maintained in an ice bath. Nuclei were resuspended at 4×10^7/mL (ca. 200 μg of DNA/mL) of cold digestion buffer (15 mM Tris, pH 7.4/0.5 mM dithiothreitol/5 mM $MgCl_2$/15 mM NaCl/60 mM KCl/0.25 M sucrose). Aliquots of 100 μL were then added to empty tubes on ice (for endogenous nuclease reactions) or to tubes containing 1–5 μL of digestion buffer containing various DNase I concentrations. DNase I digestions proceeded for 3 min at 24 °C, and endogenous nuclease reactions were carried out at 37 °C for various time intervals. Reactions were terminated by the addition of an equal volume of stop solution (20 mM EDTA/100 mM Tris, pH 8.0/2% Sarkosyl/100 μg/mL proteinase K). Tubes were subsequently incubated overnight at 37 °C, followed by two extractions with phenol, one with phenol/chloroform/isoamyl alcohol (25:24:1) and two with chloroform/isoamyl alcohol (24:1). DNA was precipitated with 2.5 volumes of ethanol at −20 °C and was recovered by centrifugation at 10000*g* for 15 min. The pellet was dissolved in 10 mM Tris, pH 7.9/0.1 mM EDTA and was dialyzed against several changes of the same buffer at 4 °C. DNase I was obtained from Worthington and proteinase K from Boehringer Mannheim.

Restriction Enzyme Digestion and Southern Transfer. All enzymes were obtained from Bethesda Research Laboratories, and restriction digests were performed according to the supplier's recommendations, utilizing 2.5–20 units/μg DNA. Digests were separated on 0.7% or 2% agarose gels, and DNA was transferred to GeneScreen or GeneScreen Plus as previously described (Montoya-Zavala & Hamlin, 1985). DNA probes were labeled with [^{32}P]dCTP by nick-translation (Rigby et al., 1977) or by the random primer method (Feinberg & Vogelstein, 1983). Specific activity was $(1–4) \times 10^8$ dpm/μg of DNA. End-labeled size markers were prepared by kinasing 123-bp and 1-kb ladders (Bethesda Research Laboratories) or mixtures of *Hin*dIII and *Eco*RI digests of λ phage DNA. Hybridization to transfers and washing procedures were carried out as described in Montoya-Zavala and Hamlin (1985). Transfers were exposed to Kodak XAR film at −90 °C with the aid of a Du Pont Cronex intensifying screen.

RESULTS

Primary Sequence of the 5′ Flanking Region of the Chinese Hamster DHFR Gene. The hamster DHFR gene is approximately 24 kb in length and contains six exons (Milbrandt et al., 1983; Carothers et al., 1983) (Figure 1A). In preliminary mapping studies, we utilized several DNA fragments from the 5′ flanking region of the gene as radioactive probes on Northern blots of poly(A+) RNA from exponential cultures of CHOC 400. The approximate start of transcription initiation was localized to a position between the *Sma*I site at −471 and *Pst*I site at +201 relative to the ATG codon (Figure 1) (J. C. Azizkhan, unpublished results). Transcriptional start sites have recently been more accurately mapped by Chasin and his colleagues by S1 nuclease and primer extension analyses (Mitchell et al., 1986). The start sites occur predominantly at −63 and less frequently at −107 (Mitchell et al., 1986) (Figure 1B). The sequence of the region extending from the *Hin*dIII site at −1240 to +11 is shown in Figure 2.

The most significant aspects of the sequence are diagrammed in Figure 3. An ATG proximal 11-bp conserved sequence (element 1, Figures 2, 3, and 4A) shares 8/11 residues with the murine sequence and 10/11 with the human sequence. A 19-bp element at −67 to −47 (element 2, Figures 2, 3, and 4A) shares 18/19 bp with the homologous sequence in the mouse and 19/19 bp with the human sequence. This element includes major transcription start sites in the hamster gene at −63 (Mitchell et al., 1986), in the mouse gene at −55 (Farnham & Schimke, 1986), and in the human gene at −71 ± 2 bp (Chen et al., 1984). Thus, all of element 1 and a part of element 2 are included in the major transcripts in all three genes. Two 29-bp repeated sequences (elements 3, Figures 2–4) are located between −154 and −84 and lie upstream of the major transcription start site. This element is present once in the human gene but occurs 4 times in its entirety in the murine gene as part of the 48-bp repeats previously described McGrogan et al., 1985). Of the seven representatives of this element in the three genes, 18/29 bp are shared among them. The consensus sequence is shown in Figure 4B.

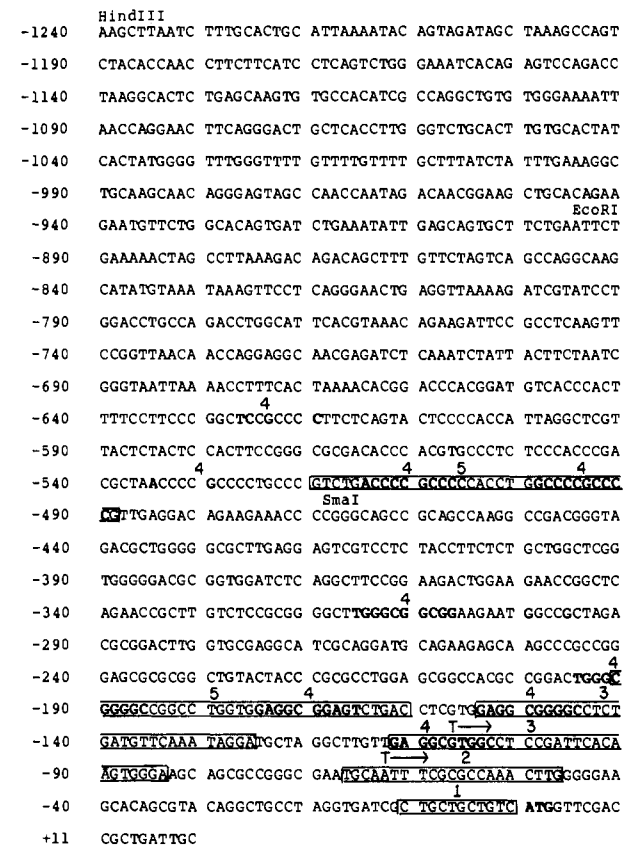

FIGURE 2: Nucleotide sequence of the 1240 bp extending upstream from the ATG codon in the Chinese hamster DHFR gene. The ATG codon at +1 is shown in bold type, as are the G/C boxes (elements 4) that conform to the Sp1 consensus binding sequence (Dynan & Tjian, 1985). The conserved elements 1, 2, 3, and 5 are outlined in boxes. The major and minor transcription start sites (Mitchell et al., 1986) are indicated with arrows above the sequence.

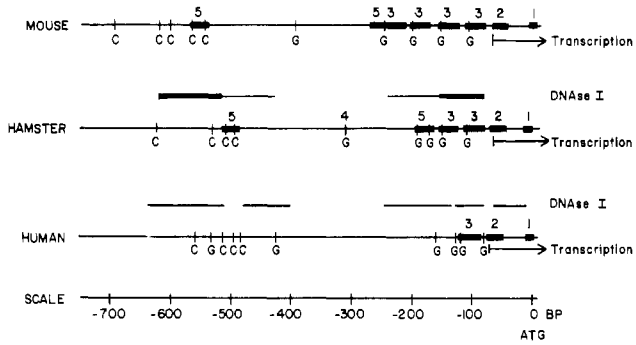

FIGURE 3: Comparison of the arrangements of homologous elements in the hamster, murine, and human DHFR genes. The conserved elements 1, 2, 3, and 5 are discussed in the text and are shown as numbered boxes on the map. The G/C boxes (elements 4) are shown as vertical lines on the map, with a G or C below each line to indicate whether the G/C box is G or C rich. The ATG codon is located at +1, and major transcription starts are indicated below each gene by horizontal arrows. The DNase I hypersensitive zones in chromatin are shown schematically above the maps of the hamster and human genes. In the hamster gene, the thick line indicates prominent hypersensitive zones and the thinner line relatively weaker zones. The zones of DNase I hypersensitivity in the human gene are shown as lines above the sequence and were taken from the data in Shimada et al. (1986).

In the hamster gene, four promoter proximal G/C boxes (elements 4, bold type, Figure 2) are clustered in the −195 to −103 region, in addition to one centered at −311 (Figure 3). These G/C boxes have the consensus G/TRGGCGG/TRGY. Another upstream cluster of G/C boxes between −629 and

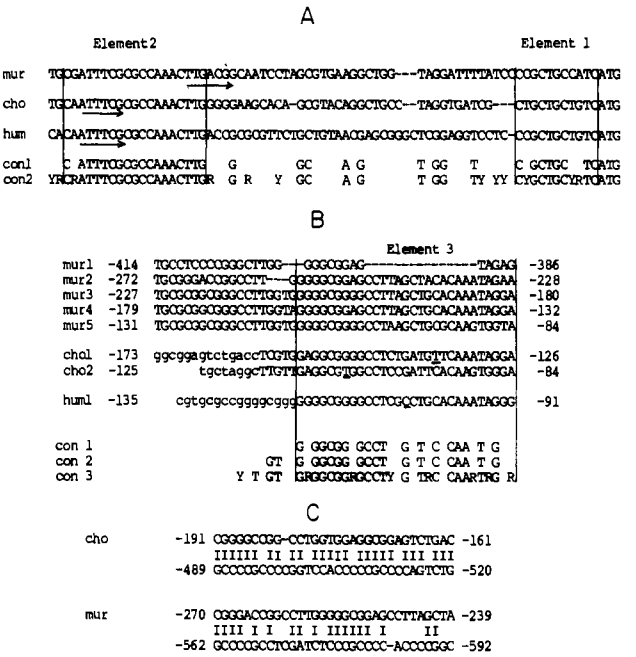

FIGURE 4: Homologous sequence elements in the hamster, murine, and human DHFR genes. Sequence data for the murine and human genes were obtained from McGrogan et al. (1985) and Chen et al. (1984), respectively. Dashes indicate deletions. (A) The sequences from −1 to approximately −70 in each gene are aligned. Element 1 is 11 bp long, element 2 is 19 bp in length, and both are bracketed by vertical lines. A conservative consensus (con 1) appears on the fourth line that shows absolute homologies, while the consensus on the fifth line (con 2) allows purine (R) or pyrimidine (Y) substitutions. The positions of major transcription start sites in element 2 are indicated by horizontal arrows below each sequence. (B) The 29-bp-repeated sequences (elements 3) in the hamster gene are aligned with homologous elements in the human and murine genes (Chen et al., 1984; McGrogan et al., 1985) (Figure 3) and are bracketed by vertical lines. The underlined residues indicate a single exception to the conservative consensus sequences indicated below. Consensus 1 (con 1) shows positions of exact homology in element 3 in all three genes, while consensus 2 shows homologies only between hamster and murine genes. Consensus 3 compares hamster and mouse and allows purine or pyrimidine substitutions. Note that the most upstream repeated sequence in the mouse (mur1) has deleted most of element 3. Note also that the five repeated elements in the murine gene share additional homologies with each other that extend further upstream from element 3. (C) The sequences of the promoter proximal inverted repeats in the hamster and murine genes are shown in the left-to-right reading sense, while the upstream copies are reversed to allow comparison of base-pairing potential.

−490 has a similar consensus when read from the opposite strand (GGGGCGGRRY). The G/C boxes are of interest because they also occur in the regulatory regions of several other genes (see Discussion) and are homologous to the consensus binding sequence for the transcriptional regulatory protein Sp1 (G/TGGGCGGRRY; Dynan & Tjian, 1985). All of the upstream G/C boxes conform to this decanucleotide consensus, while three out of four in the promoter proximal region differ only at the second position (A substituting for G). Alignment of the hamster, human, and murine DHFR genes in Figure 3 illustrates that the distribution of the G/C boxes in the three genes is quite similar. If the central G-rich box in each is disregarded, a distance of approximately 300 bp separates the upstream G/C boxes from the promoter proximal G/C box cluster. Superimposed on the G/C box clusters in the hamster gene are two almost perfect inverted repeat sequences positioned at −520 to −489 and −191 to −161 (elements 5, Figures 2, 3, and 4C). There are regions of homology to these two inverted repeated sequences in similar locations in the murine gene, although they do not align with

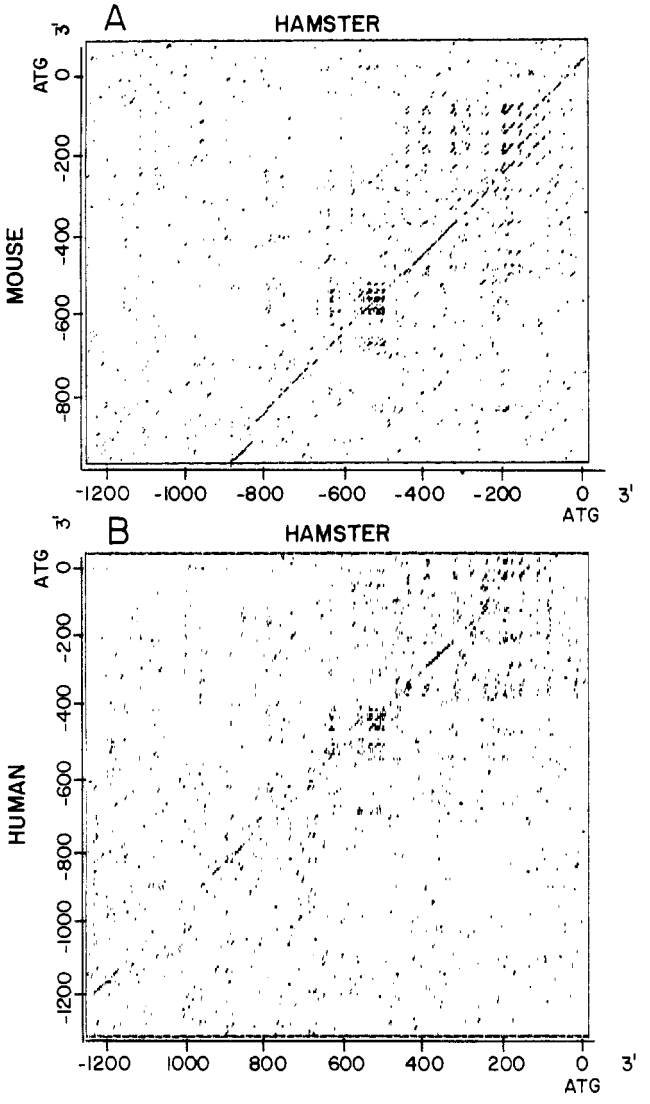

FIGURE 5: Dot homology plots (DIAGON program; Staden, 1982) comparing the hamster 5′ flanking sequence to the corresponding regions in the murine and human DHFR genes. Plots begin at approximately +40 in each gene and extend in the 5′ direction for the indicated lengths in each sequence. A single stroke on this plot represents 8 consecutive homologous bp. Note that the line will be shifted from the diagonal when a deletion occurs in one of the genes with respect to the other.

each other with the same precision as do their hamster counterparts (Figures 3 and 4C). Significant homologies to these inverted sequences could not be found in the human gene.

An extensive region of partial homology between the hamster and murine genes extends from approximately −900 to the ATG codon (Figures 3 and 5A). This homology includes the sequences upstream from the symmetrically arranged G/C boxes, in a region that may encode a second message in the mouse and hamster that is transcribed in the opposite direction (Crouse et al., 1985; Mitchell et al., 1986) (see Discussion). When the human and hamster genes are compared (Figure 5B), stretches of homology are also detected but are less extensive than between mouse and hamster. A computer analysis detects several open reading frames in the hamster gene that read in a direction opposite to the direction of the DHFR gene. These are located at −838, −771, −599, and −270 in the hamster gene. When compared to the murine and human 5′ flanking sequences (McGrogan et al., 1985; Chen et al., 1984), the open reading frame beginning at −270 in the hamster sequence is also available in a similar location and extends upstream to a consensus splice site at −407. The amino acid sequences encoded in this region by the three genes are moderately homologous to one another (75–80% if conservative substitutions are allowed; data not shown). However, none of the peptides encoded in any of the three reading frames in either direction in the hamster sequence corresponds significantly to any published sequence in the Dayhoff protein data bank.

Nuclease Sensitivity of the DHFR 5′ Flanking Region. In preliminary experiments, we utilized several cloned fragments to scan the chromatin throughout the 24-kb CHO DHFR gene for hypersensitivity to endogenous nuclease and to DNase I. Nuclei were isolated from CHOC 400 cultures either in exponential growth or at the G1/S boundary. After nuclease treatment, DNA was isolated and digested with either *Hin*dIII or *Xba*I.

With both endogenous nuclease and DNase I, hypersensitive sites were detected in restriction fragments containing the 5′ end of the gene. In Figure 6 (panels A and B) are shown autoradiographs of *Xba*I digests that were probed with the 1.3-kb *Eco*RI fragment shown in Figure 1B. The sizes of the fragments generated by endogenous nuclease and by DNase I are similar (with one exception, see below), but in most experiments, the overall pattern was more distinct with DNase I. The pattern of DNase I hypersensitivity in the 5′ flanking region of the gene was virtually identical in chromatin isolated from exponential cultures and from cells arrested at the G1/S boundary with aphidicolin (Figure 6, compare panels B and C). Hypersensitive DNA sites were not detected when a plasmid containing the 7.2-kb *Xba*I fragment was subjected to limited DNase I digestion and was similarly analyzed (data not shown). Therefore, DNase I hypersensitivity in this region is a consequence of chromatin configuration and not sequence specificity of the enzyme.

With both DNase I and endogenous nuclease, bands were observed at approximately 7.0–5.8 kb and at 4.0 kb, with a cluster of fragments smaller than 2 kb. Since the 1.3-kb *Eco*RI probe is located in the right end of the 7.2-kb *Xba*I fragment, the smallest fragments must map closest to the 5′ end of the gene, and the 4.1-kb fragment maps approximately 3.2 kb upstream from the ATG codon (see maps, Figures 1B and 3). The diffuse bands in the 7.2–5.9-kb range probably arise from the hypersensitive sites located within the region covered by the 1.3-kb *Eco*RI probe, since this probe will bind to both the small and the large fragments generated by DNase I cuts within this region. Alternatively, fragments in this size range could arise from nuclease-hypersensitive sites located at the extreme left end of the *Xba*I fragment. When the 20-kb *Hin*dIII fragment that spans the 3′ end of the gene was probed with a *Hin*dIII/*Eco*RI fragment (Figure 1A), no prominent fragments were consistently observed with either nuclease (Figure 6, panel D; data for endogenous nuclease not shown). Similar negative results were obtained with other combinations of probes and restriction enzymes that illuminate the body of the gene (data not shown).

The only detectable difference in cutting pattern between endogenous nuclease and DNase I in the 5′ region of the gene was the presence of a prominent band at 3.2 kb in endogenous nuclease digests (arrow, Figure 6A). This band is not observed in the control sample in which magnesium ion in the digestion buffer was chelated with EDTA prior to incubation at 37 °C (Figure 6, panel A), and its amount does not vary with incubation time. This band may therefore be either an artifact of the subsequent restriction digestion with *Xba*I (e.g., an altered recognition specificity) or may result from a second endogenous nuclease activity that approaches a limit very

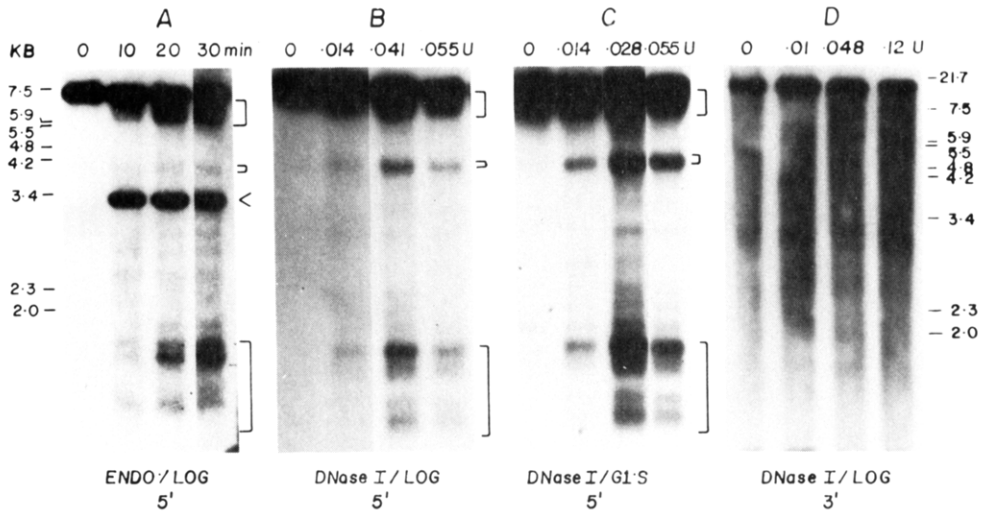

FIGURE 6: Nuclease hypersensitivity in the 5′ flanking region of the Chinese hamster DHFR gene. Brackets to the right of each panel indicate nuclease hypersensitive zones. (A) Nuclei were prepared from exponential CHOC 400 cultures as described under Materials and Methods and were taken up in ice-cold nuclease buffer (25 μg/100 μL of reaction volume). At time zero, samples were incubated at 37 °C to allow digestion by endogenous nuclease. Reactions were quenched with SDS and EDTA at the indicated times, and the DNA was purified as described. After digestion with *Xba*I, approximately 1 μg of each sample was separated on a 0.7% agarose gel, and the digests were transferred to GeneScreen. The transfer was hybridized with the 1.3-kb *Eco*RI fragment that maps in the right end of the 7.2-kb *Xba*I fragment B containing the 5′ end of the gene (Figure 1B). Size markers to the left are the *Hin*dIII fragments of λ DNA. (B and C) Nuclei from exponential (panel B) or G1/S (panel C) cultures were isolated as described above and were added to tubes containing the indicated DNase I concentrations in micrograms/100 μL (25 μg of DNA/100 μL of reaction volume). The tubes were then incubated at 24 °C for 3 min, and reactions were quenched with SDS and EDTA. DNA was digested and analyzed exactly as described above for panel A. The probe was the 1.3-kb *Eco*RI fragment from the 5′ end of the gene (Figure 1B). (D) Nuclei were isolated and were digested with the indicated DNase I concentrations. DNA was purified and digested with *Hin*dIII and was transferred to GeneScreen as above. The 20-kb *Hin*dIII fragment C, which overlaps the 3′ end of the DHFR gene, was probed with a 500-bp *Hin*dIII/*Eco*RI fragment mapping just inside of the last exon (Figure 1A).

rapidly [e.g., topoisomerase II; Liu (1983)]. The former explanation seems unlikely, since the control sample, which does not contain this fragment, was digested with *Xba*I under the same conditions as the other time points in this experiment. Furthermore, while we *have* observed altered recognition specificities with the enzyme *Eco*RI when DNA was isolated from nuclei prepared in sucrose without subsequent dialysis, we have not observed this phenomenon with the enzyme *Xba*I.

In order to map the DNase I hypersensitive sites in the 5′ flanking region of the gene more accurately, we examined DNase I/*Xba*I digests using a 250-bp *Eco*RI fragment that maps to the right of the ATG codon in the gene and lies 150 bp upstream from the 3′ *Xba*I site (see map, Figure 1B). Any novel fragment larger than 400 bp that is detected by this probe must therefore map to the left of the probe. In 2% gels, with a 123-bp ladder as internal standard, the small fragments are estimated to be in the range of 430–492 bp (weak), 800–885 bp (strong), 885–1008 bp (weak), 1168–1291 bp (weak), 1291–1353 bp (strongest). These sites therefore map between −625 and +50 in the gene (Figures 1A and 3). The smallest band, which maps in the first intron of the gene at approximately +333, is normally not observed in 0.7% gels. The diffuse band at 1291–1353 is usually stronger early in the course of DNase I digestion (i.e., at low enzyme concentration), while the zone at 800–885 bp becomes strongest at higher levels of digestion (e.g., see Figure 6). In all experiments, there is clearly an insensitive zone 150–200 bp in length that is centered at −330 between the two symmetrical hypersensitive regions.

In 0.7% gels, the DNase I/*Xba*I digests appear to be almost identical whether probed with the 1.3-kb or 250-kb *Eco*RI fragments (compare Figure 6, panel B to Figure 7, panel A). A notable exception is the diffuse cluster of bands migrating just below the full-length 7.2-kb *Xba*I fragment that was detected with the 1.3-kb probe. This cluster is not detected with the smaller probe and must therefore arise from DNase I cutting sites that are overlapped by the 1.3-kb probe but not by the 250-bp *Eco*RI fragment. From the end-labeled 1-kb ladder, we estimate the most upstream DNase I hypersensitive site to be 4.1 kb from the 3′ *Xba*I site. This site therefore lies 3.2 kb upstream from the ATG codon in the DHFR gene (Figure 1B). The 250-bp *Eco*RI probe also detects a minor band at about 6.0 kb that appears in all lanes (asterisk, Figure 7A). This band probably represents an *Xba*I restriction fragment that cross-hybridizes only with the smaller probe, since it is present in the control lane that received no DNase I, and is not detected in endogenous nuclease digests that were probed with the 1.3-kb *Eco*RI fragment (Figure 6A).

The positions of the DNase I hypersensitive zones relative to conserved sequence elements in the 5′ flanking region of the DHFR gene are indicated in Figure 7 and on the map in Figure 3. The transition from strong to weak DNase I hypersensitivity in the region proximal to the DHFR gene occurs approximately at the position of element 5 (half of the inverted repeat sequence, Figure 3). The strong hypersensitivity in this region ends approximately at the major DHFR transcription start site at −63 bp. The weak sites in both the upstream and downstream regions overlap at least two G/C boxes in each case. The symmetry of the cutting pattern between −600 and −75 bp therefore coincides with the placement of the G/C boxes and/or the inverted repeated sequences (elements 5).

The pattern of DNase I cutting in the human DHFR gene is also shown in Figure 3, where the bars above the map represent zones of DNase I hypersensitivity described by Shimada et al. (1986). From this comparison, the chromatin configuration in the upstream regions of the human and hamster genes appears to be similar with respect to the underlying conserved sequence elements, although the degree of hypersensitivity of individual sites varies somewhat between the two genes. In addition, a hypersensitive site in the human DHFR gene centered at −20 is not observed in the chromatin of the hamster gene. To our knowledge, DNase I hypersen-

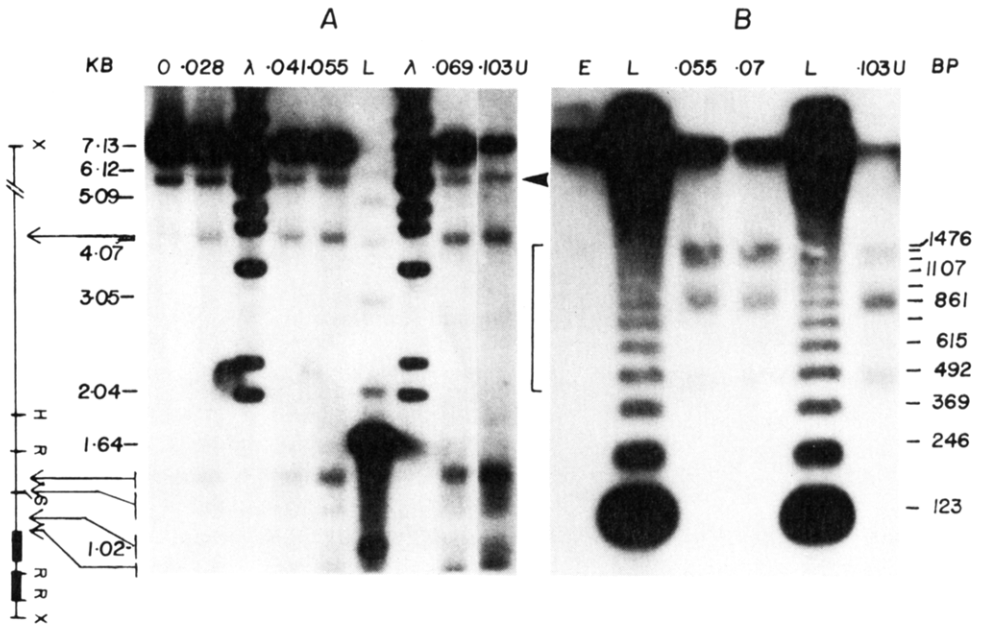

FIGURE 7: Zones of DNase I hypersensitivity in the 5′ flanking region of the DHFR gene. (A) Nuclei were prepared from a G1/S phase culture of CHOC 400 cells and were treated with the indicated DNase I concentrations exactly as in the legend to Figure 6. DNA was purified and digested with *Xba*I. Digests were separated along with a ^{32}P-end-labeled 1-kb ladder (L) and an end-labeled standard prepared from a mixture of *Hin*dIII- and *Eco*RI-cut λ DNA (sizes top to bottom: 9.5, 7.5, 5.9/5.5, 4.8, 4.2, 3.4, 2.3, and 2.0 kb). After transfer to GeneScreen, the blot was probed with the 250-bp *Eco*RI fragment shown in Figure 1B. The map to the left shows the locations of the DNase I hypersensitive zones within the *Xba*I fragment that contains the 5′ region of the DHFR gene, where black boxes indicate the first two exons. The arrowhead to the right of panel A indicates a cross-hybridizing *Xba*I fragment that is detected in all samples, including the control that received no DNase I. (B) A portion of the DNase I/*Xba*I digest from A above was separated on a 2% agarose gel, along with a 123-bp end-labeled standard (L). The transfer was probed with the 250-bp *Eco*RI fragment as in A above. The bracket to the left of the panel indicates the DNase I hypersensitive region that lies immediately upstream from the first exon of the DHFR gene.

sitivity experiments on the murine gene have not been reported.

Discussion

We have identified at least four highly conserved elements that are similarly arranged with respect to one another in the 5′ flanking regions of all three mammalian genes that have been sequenced (Chen et al., 1984; McGrogan et al., 1985; Mitchell et al., 1986) (Figure 3). An 11-bp promoter proximal sequence (element 1) of unknown function and a 19-bp conserved sequence (element 2) coinciding with the major transcription start sites are present in all three genes. Thus, all of element 1 and at least a part of element 2 are contained in the major transcripts. The major transcription start sites in the hamster and human genes at −63 and 71 ± 2, respectively (Mitchell et al., 1986; Chen et al., 1984), coincide with a CAA element (Figure 4A). Interestingly, in the murine gene, the corresponding CAA in element 2 is replaced with a CGA at −71 (Figure 4A), and a major transcription start site in this region has been detected at −55, near a second CAA element at −60 (Dynan et al., 1986). This sequence may therefore be involved in fixing the position of the major cap site of these messages.

A 29-bp conserved sequence (element 3, Figure 4B) is represented twice in the Chinese hamster gene, once in the human gene, and four times in the murine gene (as part of the 48-bp-repeated sequences previously identified (Farnham & Schimke, 1985). In all three genes, each of the 29-bp elements contains one copy of the G/C box. The promoter proximal copy of the 29-bp element in each gene bears a relatively fixed relationship to a major transcription start site, which lies 19–28 bp downstream and therefore could be involved in positioning the cap site of the message, possibly in conjunction with element 2. Element 3 could therefore replace the TATA box that has been implicated in determining the transcription start site in other genes [e.g., see Corden et al. (1980) and Grosveld et al. (1981)], but which is not found in the hamster, human, or murine DHFR genes.

The G/C boxes (elements 4) are arranged in all three genes in a symmetrical pattern whose center is displaced from the major transcription start site in each case by the number of 29-bp repeated elements in the gene (Figure 3). Almost all of the G/C boxes conform to the consensus binding sequence for the Sp1 protein, which has been shown to increase the rate of in vitro transcription of the murine DHFR and SV40 early gene (Dynan et al., 1986). This sequence (or the simpler version GGGCGG) occurs in the 5′ flanking region of several genes, including herpes simplex thymidine kinase (McKnight & Kingsbury, 1982), hamster HMG CoA reductase (Osborne et al., 1985), human adenosine deaminase (Valerio et al., 1985), human superoxide dismutase (Levanon et al., 1985), murine HPRT (Melton et al., 1984), chicken U1 RNA (Roebuck & Stumph, 1985), and the SV40 promoter (Fromm & Berg, 1982). Furthermore, it has recently been shown in DNase I footprinting experiments that the G/C boxes in the murine DHFR gene bind the Sp1 protein, albeit with different apparent affinities at each site (Dynan et al., 1986). This finding suggests that other more subtle local sequence elements may be recognized by the Sp1 protein.

The inverted and symmetrical arrangment of the G/C box clusters in the three genes suggests that the upstream elements may serve a similar role in the transcription of a second gene transcribed in a direction opposite to the DHFR gene. However, in the hamster and murine DHFR genes, divergent transcripts have been detected that initiate at −195 and −245 to −305, respectively, relative to the ATG codon (Mitchell et al., 1986; Crouse et al., 1985). These observations argue against the presence of a promoter in the region upstream from the 5′ G/C box cluster and suggest that the 29-bp-repeated sequences (elements 3) act as bidirectional promoters for both the DHFR gene and the divergent gene (Mitchell et al., 1986;

Crouse et al., 1985). This suggestion is consistent with the observation that the Sp1 consensus binding sequence in the promoter region of the thymidine kinase gene is functional in both orientations (McKnight et al., 1984). By contrast, in the human gene, an opposite-strand transcript has been detected that initiates at about −672 (Chen et al., 1984). Thus, the promoter of this upstream gene in the human system appears to be in the same location relative to the upstream G/C boxes as the DHFR promoter is to the downstream G/C boxes (Figure 3).

The striking conservation of the upstream G/C boxes in all three mammalian DHFR genes, as well as their homology to the Sp1 binding sequence, argues that they are functional and necessary for transcription. This raises a question as to the organization of this upstream region in the hamster and mouse. In both systems, the divergent transcripts that initiate at the same promoter as the DHFR messages extend upstream for 4 kb and 14 kb, respectively. This places the upstream Sp1 binding elements in the middle of these transcripts, possibly within an intron in both genes (Crouse et al., 1985; Farnham & Schimke, 1986; Mitchell et al., 1986). While we cannot presently explain the rather complex organization of this region, it is formally possible that a third gene positioned within the intron of the divergent gene initiates transcription near the G/C boxes and is also transcribed in a direction opposite to the DHFR gene. A similar arrangement has been observed in the yeast mitochondrial genome, in which the coding sequence for a splicing protein is located within an intron in the cytochrome B gene (Lazowska et al., 1980). In addition, it has recently been shown that the coding sequence for a cuticle protein is located within the intron of the *Gart* gene in *Drosophila* (Henikoff et al., 1986).

Superimposed on the more obvious individual conserved elements discussed above is a long region of homology between the three mammalian DHFR genes extending from the ATG codon in each gene to approximately −900 bp upstream (Figure 5). An analysis of the upstream region (opposite reading sense) of the hamster gene detects a potential coding sequence extending from −270 to −407 that coincides in relative location to open reading frames in the other two genes. The amino acid sequences are 75–80% homologous, if conservative replacements are allowed, suggesting that these polypeptides may be expressed. They would presumably be encoded by the divergent transcripts that initiate in the bidirectional promoters of the hamster and murine DHFR genes (Mitchell et al., 1986; Crouse et al., 1985; Farnham & Schimke, 1986). It is noteworthy, however, that no divergent transcripts initiating in the region of element 3 in the human gene have been detected (Chen et al., 1986).

Our studies on the nuclease hypersensitivity of chromatin in the 5′ flanking region of the Chinese hamster DHFR gene detect a complex pattern of digestion in the 500 bp extending from approximately −100 to −600 relative to the ATG codon. A marked symmetry in the hypersensitivity pattern suggests a relationship between the symmetrical G/C box array (including the inverted repeat elements 5) and the chromatin configuration in this region. The two most hypersensitive zones are positioned over the upstream and downstream G/C box clusters, with weaker zones located more centrally in the symmetrical sequence. The pattern of DNase I hypersensitive sites in the Chinese hamster DHFR gene have also been mapped recently by Mitchell et al. (1986). In their study, a very similar pattern of hypersensitivity was detected in the 5′ flanking region, but the pattern is shifted by approximately 50 bp in the 3′ direction relative to our map. In the studies of Mitchell et al., the hypersensitive sites in this region were mapped relative to an upstream *Hin*dIII site that they placed at −1283 relative to the ATG codon (see Figure 1B). However, our sequence extends further upstream to include this *Hin*dIII site and fixes it at −1239, accounting for the difference in placement of the hypersensitive sites in our two studies.

With the exception of a hypersensitive site positioned at approximately −20 relative to the ATG codon in the human gene, the overall pattern of DNase I digestion in the hamster gene is almost identical with respect to the position of hypersensitive zones (Shimada et al., 1986) (Figure 3). Apparent in both the human and hamster genes is a nuclease-insensitive zone of 150–200 bp in length that is centered between the upstream and downstream G/C clusters. Shimada et al. (1986) have presented evidence that this region is protected from DNase I by the presence of a protein or proteins.

It is notable that both the human and hamster genes display weak DNase I hypersensitive sites located similarly within the first intron (Figure 1B) (Mitchell et al., 1986; Shimada et al., 1986). It will be of interest to learn whether the murine gene also displays such a site. In addition, a relatively strong DNase I cutting site is observed approximately 3.2 kb upstream from the CHO DHFR ATG codon. As discussed above, the position of this site is puzzling, since the upstream gene in the hamster is thought to initiate transcription at approximately −195 relative to the ATG codon and to extend upstream for 4 kb (Mitchell et al., 1986). This site would therefore be located within the body of the upstream gene. A more thorough analysis of this upstream region is required before the significance of this site with respect to transcription can be understood.

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Registry No. DNase I, 9003-98-9.

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Changes in Retinal Position during the Bacteriorhodopsin Photocycle: A Resonance Energy-Transfer Study†

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ABSTRACT: Distances from fluorescent lipid probes to the retinal chromophore of bacteriorhodopsin incorporated into asolectin vesicles have been measured with resonance energy transfer. Steady-state fluorescence is used to find the distance of closest approach from fluorescent lipid probes to ground-state retinal. Phase modulation of resonance energy transfer is used to find distances for retinal in bacteriorhodopsin's M intermediates. This latter technique uses an actinic light source to drive the photocycle while absorbance of a photocycle intermediate and fluorescence due to energy transfer to that intermediate are monitored with phase-sensitive detection. The photocycle intermediate concentration is varied by changing the frequency of modulation of the actinic light. Previous measurements of distances to the M intermediates using this technique [Hasselbacher, C. A., Preuss, D. K., & Dewey, T. G. (1986) *Biochemistry 25*, 668-676] are extended by use of several different fluorescent probes allowing labeling of either the bilayer surfaces or the interior. This provides a more detailed mapping of retinal's position. Analysis of the phase modulation data is improved in several ways to obtain accurate distances for the M intermediates. These distances are compared to ground-state distances. Results show that retinal is buried more deeply in the protein when bacteriorhodopsin is in its ground state than in either M state. There is also a significant change in retinal location between the slow-decaying and fast-decaying M intermediates. These large changes in retinal's position during the photocycle demonstrate the flexibility of monomeric bacteriorhodopsin.

Bacteriorhodopsin is a membrane-bound protein synthesized by the archaebacterium *Halobacterium halobium*. It actively transports protons across the bacterial membrane and maintains the proton gradient required for metabolic processes during oxygen deprivation. The proton pumping process is initiated by the absorption of light by bacteriorhodopsin's

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