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Human Argininosuccinate Synthetase Minigenes Are Subject To Arginine-Mediated Repression but Not To *trans* Induction

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The human argininosuccinate synthetase locus is subject to metabolite-mediated repression by arginine in some cultured cell lines. To gain insight into the mechanism underlying this regulation, chloramphenicol acetyltransferase (CAT) minigenes under the transcriptional control of the human argininosuccinate synthetase promoter were constructed and tested for regulation. When the minigenes were introduced into RPMI 2650 cells, a human cell line that shows sixfold regulation of the argininosuccinate synthetase gene, CAT expression was repressed three- to fivefold when arginine was present in the culture medium. A minigene containing only 149 base pairs of 5′-flanking sequence was expressed at similar levels and regulated to the same degree as one having approximately 3 kilobases of 5′-flanking sequence. Therefore, the *cis*-acting sequences required for the arginine-mediated repression are likely to be located within the region of the transcription initiation site. The arginine-mediated repression of the CAT minigenes was not observed in canavanine-resistant variants of RPMI 2650 cells, and therefore they showed the appropriate cell-type specificity. Cultured cells having 200-fold-increased levels of argininosuccinate synthetase can be selected by growth in medium containing the arginine analog canavanine. It was previously demonstrated that the increased expression of argininosuccinate synthetase in canavanine-resistant human lymphoblasts was due to a *trans*-acting mechanism. To gain further support for a *trans*-acting mechanism, we tested our CAT minigenes for the *trans* induction in canavanine-resistant variants of RPMI 2650 cells. Transfection of the CAT minigenes into RPMI 2650 cells and canavanine-resistant variants of this cell line yielded no difference in transient CAT expression. Furthermore, cloned canavanine-resistant variant cells having integrated copies of the CAT minigenes expressed CAT at similar levels as compared to the parental cell lines. Since these cell lines do exhibit arginine-mediated repression of CAT but not *trans* induction, these data indicate that the arginine-mediated repression is a regulatory event that occurs independently of the *trans* induction.

There are many examples of eucaryotic genes that are regulated in *trans* by hormones (13, 16, 25, 26), metals (3, 13, 25, 29), and viral or cellular gene products (10, 22). In most cases studied thus far, regulation occurs at the level of gene transcription such that a rapid change in transcription is concomitant with addition of the factor mediating the regulation. It has been shown for glucocorticoid hormone regulation of both cellular and viral genes that a hormone-receptor complex exerts its effect by binding to regions 5′ to or within the regulated gene and thereby stimulating transcription. The *cis*-acting sequences involved in the regulation have been mapped by gene transfer with cloned DNA and by various physical techniques that are based on stable interactions between the *cis*-acting element and the regulator (20, 21). Indirect evidence for cellular *trans*-acting regulators has been obtained by using DNA competition assays (17, 28). In these experiments, saturating amounts of cloned DNA containing the *cis*-acting element are included in the expression assay, thereby titrating out the factor involved in the regulation. In most cases studied thus far, the regulatory factor exerted a positive effect on the transcription rate of the regulated gene.

There are few examples of eucaryotic genes that are under the control of a negative-acting regulator. The best-studied example of a eucaryotic repressor is simian virus 40 (SV40) T-antigen which represses the transcription of its own gene during the shift from the early phase to the late phase of the viral life cycle (33). The large T-antigen binds to several sites within the SV40 early promoter region, with the site having the greatest affinity lying downstream from the early gene transcription initiation site (18). The Chinese hamster HMG coenzyme A (CoA) reductase gene is also subject to negative-feedback control that is mediated by low-density lipoproteins, mevalonate, and 25-hydroxycholesterol (14, 24). This regulation occurs at the level of gene transcription, and a rapid decrease in transcription is concomitant with the addition of these mediators to the culture medium. The SV40 large-T-antigen gene and the HMG CoA reductase gene have promoters that are very G-C-rich and have the sequence CCGCCC repeated several times within this region. In SV40 the transcription factor Sp1, which is important in promoter selection, binds to this repeated hexanucleotide sequence (4).

The human argininosuccinate synthetase gene is subject to metabolite-mediated repression by arginine in some cultured cell lines. Argininosuccinate synthetase plays a role in urea synthesis in liver and is involved in arginine biosynthesis in nonhepatic tissues. The negative-feedback regulation was first described in rodent and human cells by Schimke (27) and later in other mammalian cell lines such as hamster fibroblasts (9), human lymphoblasts (11), and a human squamous cell carcinoma cell line, RPMI 2650 (30). Cells grown in medium in which citrulline, a substrate for the argininosuccinate synthetase reaction, is substituted for arginine have 3- to 150-fold-higher levels of argininosuccinate synthetase activity relative to cells grown in medium containing arginine. It was shown that citrulline is not an activator of the argininosuccinate synthetase locus (27).

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Argininosuccinate lyase, which converts argininosuccinate into arginine, is not subject to arginine-mediated repression (11, 30). This indicates that the feedback regulation is specific for the argininosuccinate synthetase locus. It was previously shown that the arginine-mediated repression occurs at the pretranslational level (31). The human argininosuccinate synthetase gene has been cloned and characterized (5). DNA sequence analysis of the promoter region demonstrated that it was 80% G-C-rich and contained several nucleotide stretches that resemble the repeated hexanucleotide sequence present in the promoters of the SV40 early gene and the HMG CoA reductase gene.

Argininosuccinate synthetase expression is also altered in cultured cells resistant to canavanine, a toxic arginine analog (12, 30). Canavanine-resistant (Can^r) cells can have up to 300-fold-higher levels of argininosuccinate synthetase as compared with wild-type cells. The increased expression of argininosuccinate synthetase in Can^r cells is regulated at the pretranslational level and is not due to gene amplification (31). Once established, the Can^r phenotype is stable, and the argininosuccinate synthetase locus is not subject to arginine-mediated repression in the Can^r cells. Previous data have indicated that the increased levels of argininosuccinate synthetase in canavanine-resistant human lymphoblasts were due to a *trans*-acting mechanism (S. Freytag, T. Su, W. O'Brien, and A. Beaudet, unpublished data). Canavanine-resistant variants of a human lymphoblast cell line having an S1 nuclease-detectable defect in one argininosuccinate synthetase allele were isolated and shown to have at least 80-fold-higher levels of argininosuccinate synthetase as compared with wild-type lymphoblasts. S1 nuclease analysis of RNA from the canavanine-resistant lymphoblasts showed that both the normal and mutant argininosuccinate synthetase mRNAs were present in equally increased amounts as compared with the wild-type lymphoblasts. Since the lymphoblasts were isolated from the father of a child having complete argininosuccinate synthetase deficiency, the mRNA from the mutant allele could not have coded for a functional protein. Therefore, the mutant allele was not subject to the selective pressure imposed by the canavanine selection. These data strongly indicated that the canavanine-resistant phenotype was due to a *trans*-acting mechanism that affected the expression of both argininosuccinate synthetase alleles.

We constructed a series of chloramphenicol acetyltransferase (CAT) minigenes under the transcriptional control of the human argininosuccinate synthetase promoter to map the *cis*-acting sequences necessary for the arginine-mediated repression and *trans* induction and to use as probes to identify and characterize cellular *trans*-acting regulators. In this report we demonstrate that the CAT minigenes are repressed by arginine in a fashion similar if not identical to that of the natural human argininosuccinate synthetase gene; however, they are not expressed at high levels in canavanine-resistant cells.

MATERIALS AND METHODS

Cell culture. All cell lines were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.4), 2 mM glutamine, 100 μg of streptomycin per ml, 100 U of penicillin per ml, and 10% fetal bovine serum. RPMI 2650 cells, canavanine-resistant variants of this cell line (Can^r-1), and RJK88 cells were obtained from William O'Brien (Baylor College of Medicine). Cell monolayers were grown in a humidified incubator containing an atmosphere of 90% air and 10% CO_2. The medium was replaced every 3 days unless indicated otherwise.

Transfections were carried out by the $CaPO_4$ precipitation method of Parker and Stark (19). Typically 10^6 cells were plated out the day before transfection. At 5 h after the addition of the $CaPO_4$-DNA precipitate, the cells were subjected to shock with 15% glycerol for 1 min. The cells were then subdivided and plated at a density of 1×10^4 to 5×10^4 cells per cm^2 in selective medium. Selection for expression of RSVneo was performed in DMEM containing 1 mg of G418 (GIBCO Laboratories, Grand Island, N.Y.) per ml. In all selections, cloned cell lines were established 12 to 14 days after the selection was applied, and cells were propagated in selective medium for at least two additional weeks.

Construction of minigenes. Argininosuccinate synthetase-CAT minigenes were constructed by established procedures (15). All human DNA present in the minigenes was derived from the recombinant bacteriophage AS17 (5) or from subcloned fragments of this genomic clone. All CAT minigenes were constructed with pSV0cat (7). The contents of minigenes are described in the text. The SV40 enhancer region contained in pmgAS-14 and pmgAS-15 was derived from a 413-base-pair (bp) *Acc*I-*Nco*I restriction fragment of pSV2gpt and contains SV40 sequences between positions 5198 and 187 as described by Reddy et al. (23). This fragment also contains pBR322 sequences between positions 2248 and 2299 as described by Sutcliffe (32). The ends of this fragment were made blunt with the Klenow subunit of *Escherichia coli* DNA polymerase, and *Bam*HI linkers were added. This fragment was then ligated into *Bam*HI sites of pmgAS-9 (making pmgAS-15) and pmgAS-10 (making pmgAS-14) after one of the two *Bam*HI sites in the original plasmids was removed.

Primer extension assays. Total cellular RNA was prepared by lysing cells in 6 M guanidinium isothiocyanate followed by centrifugation in CsCl density gradients (15). Total RNA (20 μg) were mixed with 1×10^5 to 2×10^5 cpm of an oligonucleotide primer that was 5' end labeled to a specific activity of 10^9 cpm/μg with [γ-^{32}P]ATP and polynucleotide kinase. The primer has the following sequence, which is complementary to exon 1 of the human argininosuccinate synthetase gene: 5'-CGGGCTCGGGCAGCGGTGGCA-3'. The mixture was heated to 90°C for 2 min and then placed at 68°C for 1 h. Primer extension was carried out in a volume of 50 μl having the following final concentrations: 140 mM KCl, 20 mM 2-mercaptoethanol, 1 mM each deoxyribonucleotide triphosphate, 100 mM Tris hydrochloride (pH 8.3), 150 U of RNasin (Promega Biotech) per ml, 10 mM $MgCl_2$, and 4,000 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, Md.) per ml. Reactions were performed at 42°C for 1 h. RNA was hydrolyzed in 0.1 N NaOH–10 mM EDTA at 68°C for 1 h, and the DNA was precipitated with ethanol and applied to a 12% polyacrylamide sequencing gel.

Southern blotting. Genomic DNA was prepared by lysing cells in a solution containing 10 mM Tris hydrochloride (pH 7.8), 5 mM EDTA, 0.5% sodium dodecyl sulfate; and 100 μg of proteinase K per ml at 37°C for 16 h. The DNA was extracted with phenol followed by chloroform, treated with DNase-free RNase A, and extracted again with phenol followed by chloroform. The DNA was dialyzed overnight against 10 mM Tris hydrochloride–10 mM NaCl–1 mM EDTA, pH 7.4. The DNA was subjected to digestion with *Eco*RI (4 U/μg of DNA) for 16 h and analyzed by Southern

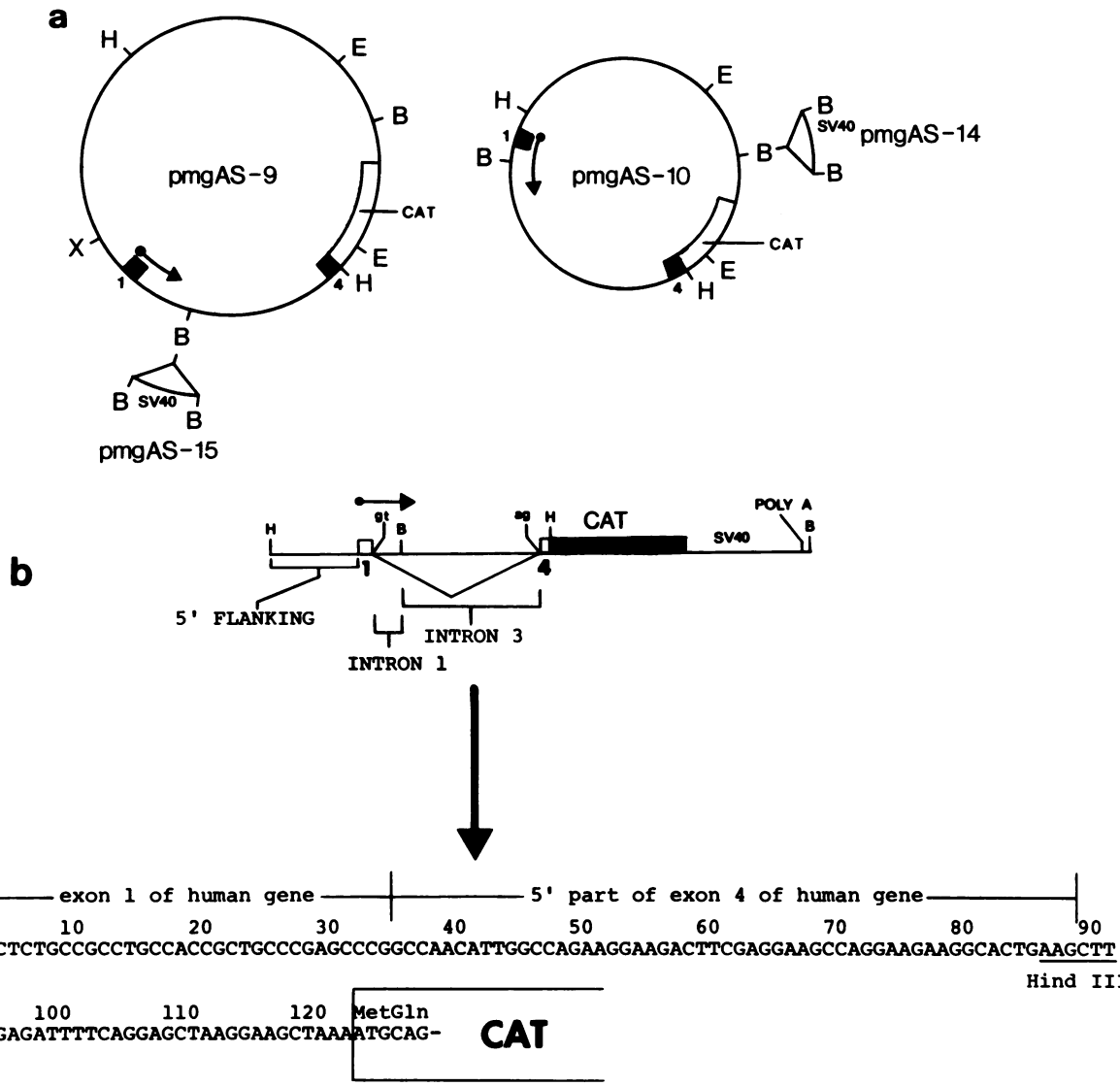

FIG. 1. Structure of human argininosuccinate synthetase-CAT minigenes. (a) All constructs were made in pSV0cat. Human argininosuccinate synthetase sequences are located in the *Hin*dIII fragment containing the solid boxes. The solid boxes represent exons from the natural human argininosuccinate synthetase gene. The CAT gene is represented by the open box. The transcription initiation site of the CAT minigenes is shown by the arrow. E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; X, *Xho*I; SV40, SV40 enhancer fragment. (b) Splicing pattern and sequence of the 5′ untranslated region of the CAT minigenes. Exons are indicated by the open boxes.

blotting, using a modification of the method previously described (6). Radioactive probes having specific activities of 1×10^8 to 6×10^8 cpm/μg were prepared by nick translation.

CAT assays. CAT assays were performed as previously described (7) with the following modifications. The final concentration of acetyl-CoA was 4 mM (34), and before the assay, the cell extracts were heated to 55°C for 5 min followed by centrifugation to remove insoluble material. The latter procedure removed an endogenous deacetylating activity. Assays in which two extracts were compared for CAT activity were performed, using an equal amount of cellular protein as assayed by A_{280}. The amount of radioactivity in all the acetylated products was determined by scintillation spectrophotometry. All assays were linear with time and protein concentration over the ranges used.

S1 nuclease assays. S1 nuclease assays were performed as previously described (5). Probes were 3′ end labeled with 500 μCi of [α-^{32}P]dCTP and 2.5 U of the Klenow subunit of *E. coli* DNA polymerase (15). The specific activities of the probes were approximately 10^7 cpm/μg.

RESULTS

Construction of argininosuccinate synthetase-CAT minigenes. To develop an understanding of the mechanism underlying the arginine-mediated repression of the human argininosuccinate synthetase gene, we constructed a series of CAT minigenes under the transcriptional control of the human argininosuccinate synthetase promoter (Fig. 1a). Minigene pmgAS-9 contains the following sequences from the natural human argininosuccinate synthetase gene (5): approximately 3 kilobases (kb) of 5′-flanking sequence, all 34 bp of exon 1, approximately 350 bp of intron 1, 1,961 bp of intron 3, and 54 bp of exon 4. These sequences were cloned

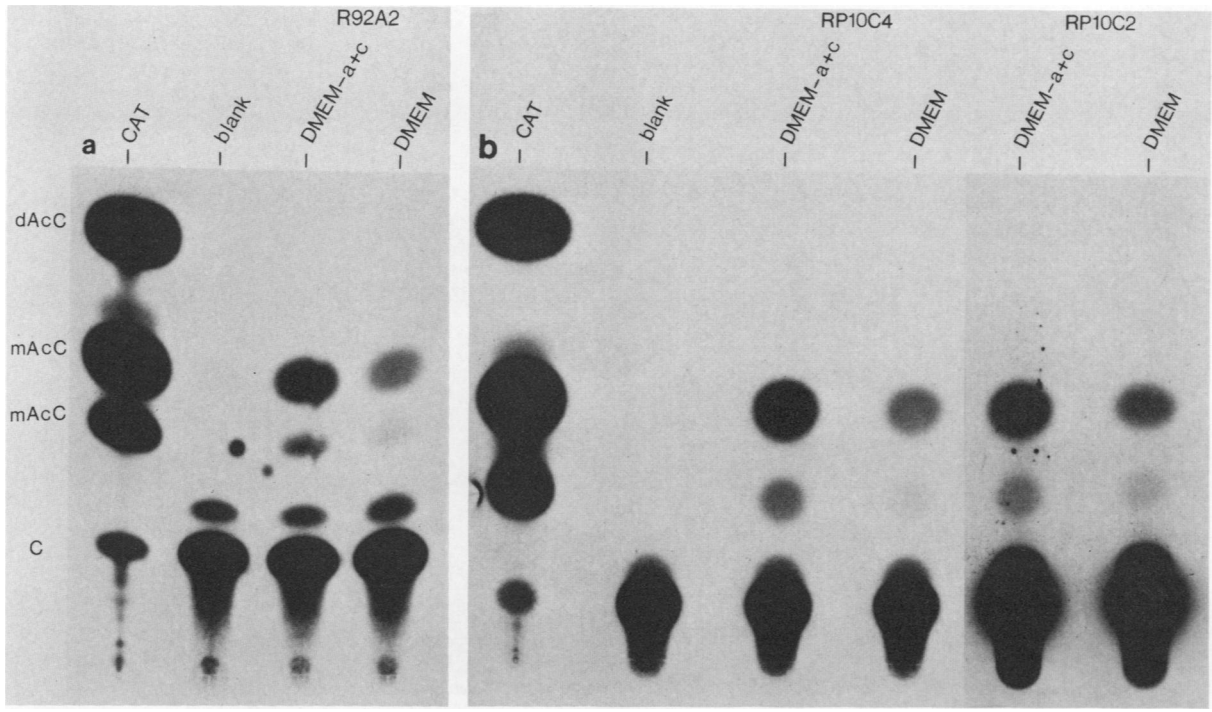

FIG. 2. Arginine-mediated repression of CAT minigenes. Cloned RPMI 2650 cell lines expressing the CAT minigenes were established by cotransfection with RSVneo followed by selection in medium containing G418. The cells were propagated in DMEM and then subcultured into DMEM−a+c or DMEM for 4 to 6 days before harvesting for CAT assays. (a) Cloned cell line R92A2 expressing pmgAS-9. CAT, CAT enzyme (Sigma Chemical Co., St. Louis, Mo.); blank, cell extract with no acetyl-CoA added. Assays were for 6 h with 1×10^6 to 2×10^6 cells. The diacetylated (dAcC) and monoacetylated (mAcC) forms of chloramphenicol (C) are indicated. (b) Cloned cell lines RP10C4 and RP10C2 expressing pmgAS-10. The first two lanes are the same as in panel a. The assays were performed as described in the legend to panel a. The X-ray exposure for RP10C2 is three times that of RP10C4.

into the *Hin*dIII site of pSV0cat (7) such that the argininosuccinate synthetase promoter would drive the expression of CAT. Minigene pmgAS-10 is similar to pmgAS-9 except it has only 149 bp of 5′-flanking sequence. Minigene pmgAS-15 is the same as pmgAS-9 except that it contains the SV40 enhancer region cloned into the *Bam*HI site in the hybrid intron. The enhancer is approximately 350 bp downstream from the transcription initiation site in this construct. Minigene pmgAS-14 is identical to pmgAS-10 except that it contains the SV40 enhancer region cloned approximately 3 kb upstream from the argininosuccinate synthetase transcription initiation site. This enhancer fragment lacks the SV40 early promoter and is cloned such that SV40 late promoter is in the opposite transcriptional orientation as the argininosuccinate synthetase promoter as determined by DNA sequencing. All minigenes contain a hybrid intron (between intron 1 and 3 joined at a *Bam*HI site) in which the 5′ splice site is provided by intron 1 and the 3′ splice site is provided by intron 3. The splicing pattern and sequence of the 5′ untranslated region of the resulting CAT mRNA are shown in Fig. 1b. Since neither exon 1 nor exon 4 of the human argininosuccinate synthetase gene contains an AUG codon, the authentic CAT initiation codon is used.

Arginine-mediated repression of CAT minigenes. Regulation of the argininosuccinate synthetase gene has been observed in human lymphoblasts (11), a human squamous cell carcinoma cell line, RPMI 2650 (30), and in hamster fibroblasts after transfection with human metaphase chromosomes (9). Although lymphoblasts show the greatest degree of regulation, these cells are poor recipients for gene transfer and were not used in these studies. RPMI 2650 cells were cotransfected with the CAT minigenes and RSVneo (8) as a dominant selectable marker conferring resistance to the aminoglycoside antibiotic G418. At 2 weeks posttransfection, multiple cloned cell lines were isolated and screened for expression of CAT. Cell lines expressing CAT were then placed in medium containing 0.6 mM arginine (DMEM) or in medium in which arginine was substituted by 0.6 mM citrulline (DMEM−a+c). After 4 to 6 days, the cells were harvested and assayed for CAT (Fig. 2). Cell lines expressing either pmgAS-9 or pmgAS-10 showed three- to fivefold less CAT activity when grown in DMEM compared with cells grown in DMEM−a+c. For example, cell line R92A2 which expresses pmgAS-9 had 4.3-fold less CAT activity when placed in DMEM (Fig. 2a). Similar results were obtained for cell lines RP10C4 and RP10C2 which express pmgAS-10 (Fig. 2b). In these cell lines CAT activity was 5.1- and 3.0-fold lower respectively, in DMEM as compared with that in DMEM−a+c. This degree of repression was very similar to that seen for the endogenous argininosuccinate synthetase gene which is repressed sixfold in the presence of arginine (30). Repression of the CAT minigenes was not due to a nonspecific effect on protein synthesis since repression was not observed in all cell lines tested (see Fig. 5). These data demonstrated that the CAT minigenes were responsive to the arginine-mediated repression and that only 149 bp of 5′-flanking sequence was necessary for regulation. In all RPMI 2650 cell lines tested thus far that have measurable CAT activity above background, we observed the arginine-mediated regulation.

Mapping transcription initiation sites in CAT minigenes. To

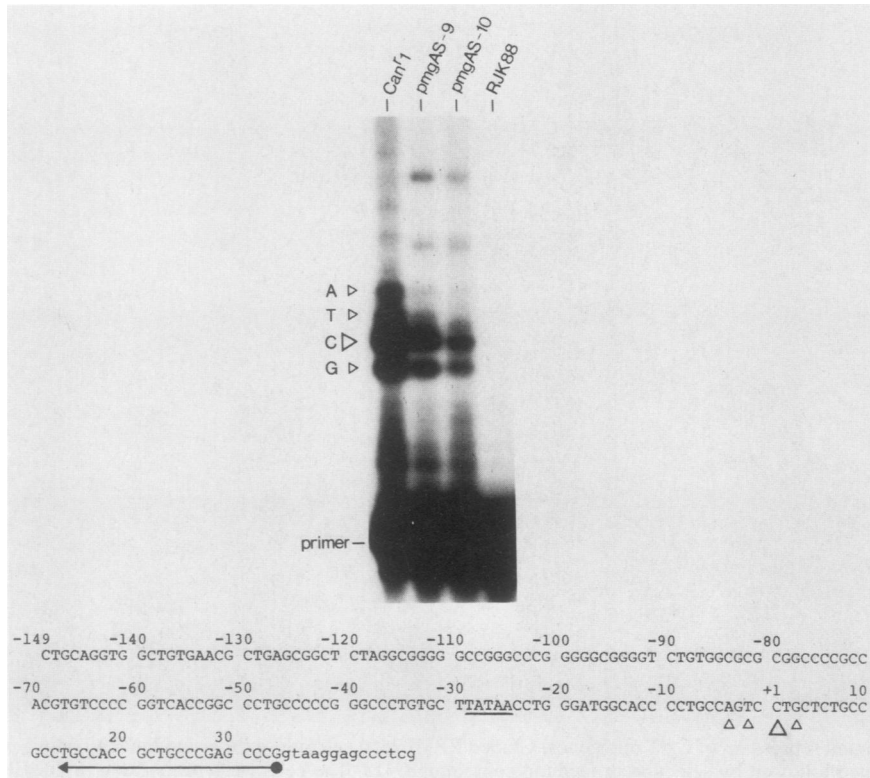

FIG. 3. Mapping transcription initiation sites in CAT minigenes by primer extension. Lanes: Can^r-1, RNA from Can^r-1 cells; pmgAS-9, RNA from RJK88 cells expressing pmgAS-9; pmgAS-10, RNA from RJK88 cells expressing pmgAS-10; RJK88, RNA from RJK88 cells. Assays were performed with 20 μg of total RNA. The sequence of the promoter region of the human argininosuccinate synthetase gene is shown. The 21-base primer is shown by the arrow. Lower-case letters are intron sequences, and upper-case letters are 5′-flanking or exon sequences.

be certain that expression of the CAT minigenes was due to the presence of the argininosuccinate synthetase promoter, we used primer extension experiments to map the transcription initiation sites (Fig. 3). The CAT minigenes were introduced into a Chinese hamster cell line, RJK88, by cotransfection with RSVneo so that a 21-base oligonucleotide complementary to exon 1 could be used as a primer. This oligonucleotide could not be used to map the cap sites of the CAT minigene mRNA in RPMI 2650 cells since this primer would also hybridize to the endogenous human argininosuccinate synthetase mRNA. Since we also have observed the arginine-mediated repression of the CAT minigenes in RJK88 cells (unpublished data), the transcription initiation sites used in these cells are likely to be identical to those used in the RPMI 2650 cells. The 21-base primer hybridizes to the 3′ end of exon 1. Two major and two minor primer extension products that are 11 to 17 bases longer than the primer itself were generated when the RNA template was from Can^r-1 cells (Fig. 3) and human liver (data not shown). These initiation sites map in a region that is 26 to 32 bases downstream from the TATAA box. The predominant initiation site (Fig. 3, C, +1) lies 29 bp downstream from the TATAA box, which is consistent with that observed from many other eucaryotic genes (2). Both CAT minigenes had identical transcription initiation sites with the predominant sites mapping to the same positions observed for the human argininosuccinate synthetase gene (Fig. 3). The CAT minigenes also had minor initiation sites that were not used in the Can^r-1 cells. These data show that the CAT minigenes used the authentic argininosuccinate synthetase transcription initiation sites and strongly indicated that the arginine-mediated repression was due to the presence of the human argininosuccinate synthetase promoter.

Structural organization of integrated CAT minigenes. Southern blot analysis was used to study the structural organization of the CAT minigenes and to quantify copy number. In the example shown (Fig. 4), genomic DNA from five cloned G418-resistant cell lines was digested with *Eco*RI and probed with RSVcat plasmid DNA. This probe was chosen since it would hybridize to CAT minigene sequences and to RSVneo sequences. These cloned cell lines were derived from an experiment in which RPMI 2650 cells were transfected with pmgAS-10 and RSVneo. The CAT minigene yielded two hybridizing *Eco*RI fragments of 4.0 and 2.13 kb (Fig. 1a). The larger fragment contained the argininosuccinate synthetase promoter region, and therefore the presence of this fragment indicates the integrity of this region in the minigene. The Southern blot demonstrated that all five cell lines had one or two copies of RSVneo and that three of the five cell lines contained the CAT minigene. CAT assays demonstrated that only cell lines RP10C2 (lane B) and RP10C4 (lane D) expressed the CAT minigene. Cell line RP10C2 had two or three copies of the minigene (based on the 2.13-kb fragment), only one of which appeared to have an intact promoter fragment. Cell line RP10C4 had three or four copies of the CAT minigene with most or all of them having intact promoter fragments. These data are consistent with the observation that RP10C4 had more CAT activity than RP10C2 (Fig. 2). Cell line RP10C5 (lane E) had what appeared to be one intact minigene, but no detectable CAT

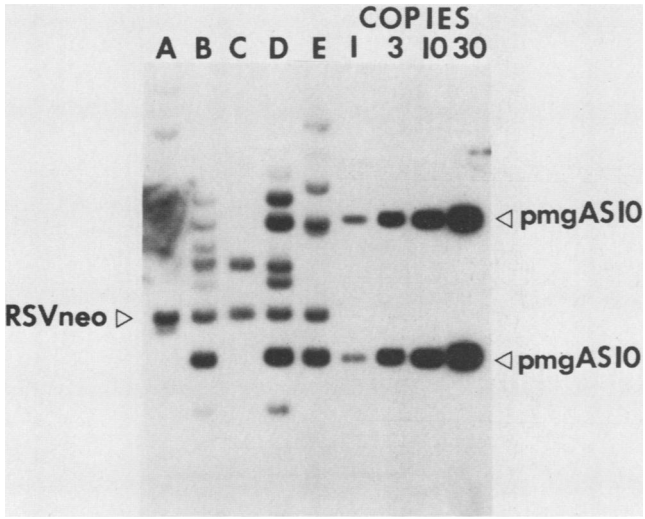

FIG. 4. Southern blot of genomic DNA from RPMI 2650 cells containing integrated copies of pmgAS-10 and RSVneo. Genomic DNA and copy controls (pmgAS-10 plasmid) were digested with *Eco*RI. The blot was probed with RSVcat plasmid DNA. Lanes A through E, Five G418 resistant clones (RP10C1 through RP10C5, respectively). The hybridizing fragments for RSVneo and pmgAS-10 are indicated.

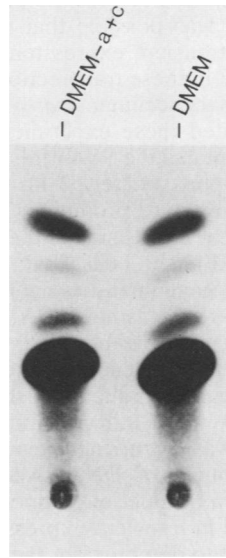

FIG. 5. Appropriate cell-specific regulation of CAT minigenes. Can^r-1 cells were transfected with pmgAS-9 and RSVneo, and cloned cell lines were tested for regulation of the CAT minigenes as described in the legend to Fig. 2.

activity was measured in this cell line. Other cell lines that express and regulate the CAT minigenes have had as many as 10 to 15 copies (data not shown).

Cell-specific regulation of CAT minigenes. It was previously shown that the human argininosuccinate synthetase gene was not subject to arginine-mediated repression in canavanine-resistant variants of RPMI 2650 cells (11, 30). To determine whether the CAT minigenes were capable of showing the appropriate cell-specific regulation, we introduced the CAT minigenes into the Can^r-1 cell line (31) using RSVneo as a selectable marker. Cloned cell lines were established, and cells were then placed in DMEM−a+c or DMEM as described above and assayed for CAT. These experiments demonstrated that the CAT minigenes were not subject to the arginine-mediated repression in any of the cloned cell lines examined since CAT activity was independent of arginine concentration in the medium (Fig. 5). Therefore, the CAT minigenes were subject to the same regulatory controls as the human argininosuccinate synthetase gene. In the example shown and in all cell lines tested, the level of CAT expression in Can^r-1 cells was in the range of that measured in the RPMI 2650 cell lines described above. Therefore, the CAT minigenes were not expressed at greatly elevated levels in the Can^r-1 cells as is the natural endogenous argininosuccinate synthetase gene.

Lack of *trans* induction with CAT minigenes. Cell variants having greatly increased expression of argininosuccinate synthetase can be selected in medium containing canavanine. It was previously shown that the increased expression of argininosuccinate synthetase in canavanine-resistant variants of human lymphoblasts was due to a *trans*-acting mechanism (Freytag et al., unpublished data; see Introduction). If the CAT minigenes contained the proper *cis*-acting sequences necessary for the *trans* induction, transfection of the CAT minigenes into canavanine-resistant cells should yield higher CAT expression as compared with that in RPMI 2650 cells. Therefore, we tested pmgAS-9, which contains approximately 3 kb of 5′-flanking sequence, for the *trans* induction in transient expression assays and in stable cell lines. Both Can^r-1 cells and RPMI 2650 cells were transfected with 20 μg of pmgAS-9, and 48 h later the cells were harvested and assayed for CAT (Fig. 6a). No difference in CAT expression was observed between the two cell lines, and these results were reproduced in several independent experiments. Similar results were obtained with pmgAS-10. Other experiments with RSVcat have demonstrated that the

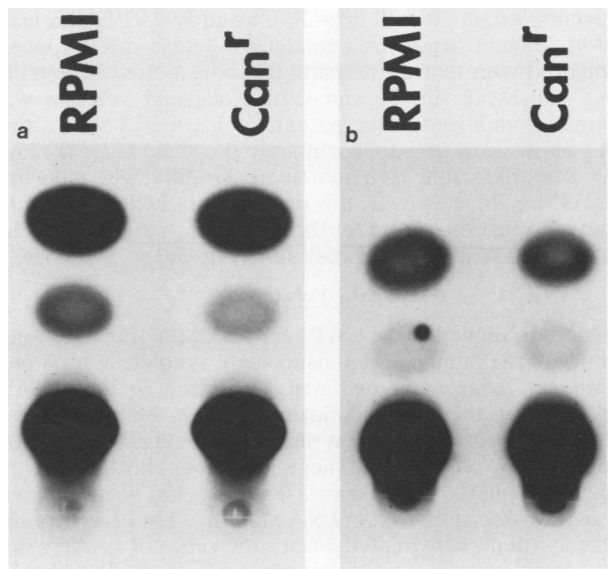

FIG. 6. Lack of *trans* induction with CAT minigenes, using transient expression assays. (a) RPMI 2650 cells and canavanine-resistant variants (Can^r-1) of this cell line were transfected with 20 μg of pmgAS-9. Forty-eight hours later the cells were harvested and assayed for CAT. The assays were for 6 h. (b) Same as in panel a except that the cells were transfected with 0.1 μg of pmgAS-14 plus 19.9 μg of carrier DNA.

two cell lines used in these studies are equally transfectable (data not shown). It was possible that the *trans* induction was not seen in the transient expression assay because the amount of DNA used in these transfections was sufficient to titrate out a necessary cellular *trans*-acting factor (28). Therefore, we repeated these experiments using minigene pmgAS-14, which contains the SV40 enhancer region cloned into a *Bam*HI site approximately 3 kb upstream from the argininosuccinate synthetase promoter (Fig. 1a). The SV40 enhancer stimulated the expression of the CAT minigenes up to 100-fold in RPMI 2650 cells (data not shown), and therefore pmgAS-14 gave measurable CAT activity even when less than 0.1 μg of plasmid DNA was used per dish. Transient expression experiments with this minigene gave results similar to those oberved when the enhancer was not present (Fig. 6b). The *trans* induction also was not seen with pmgAS-15. Although it was still possible that titration of a *trans*-acting factor was occurring even when using these greatly reduced amounts of DNA, we feel these results indicate that the *trans* induction cannot be demonstrated with these minigenes in transient expression experiments.

We next attempted to demonstrate the *trans* induction by making canavanine-resistant variants of cloned RPMI 2650 cells having integrated copies of the CAT minigenes. RPMI 2650 cells were transfected with pmgAS-9 and RSVneo, and cloned cell lines resistant to G418 were established. The cell lines were then assayed for CAT expression and screened by Southern blot analysis to identify cloned cell lines containing fewer than or equal to five copies of the CAT minigenes integrated (data not shown). Cells were then placed in medium containing 3 μM canavanine, and 2 weeks later canavanine-resistant variants were isolated from several cloned cell lines. S1 nuclease assays were used to demonstrate that the endogenous argininosuccinate synthetase gene was expressed at greatly elevated levels in the canavanine-resistant variants as compared with the wild-type cells (Fig. 7a). In the example shown, canavanine-resistant variants of cell lines R7C5 and R92A2 had at least 100-fold-higher argininosuccinate synthetase mRNA levels compared with that of the parental cells. When the level of CAT expression in the canavanine-resistant variants was compared with that in the parental cells, it was shown that CAT expression was approximately the same (Fig. 7b). We saw a reproducible threefold increase only with cell line R7C5 (Fig. 7b); however, this degree of induction was small relative to that measured for the endogenous argininosuccinate synthetase gene in this cell line (Fig. 7a).

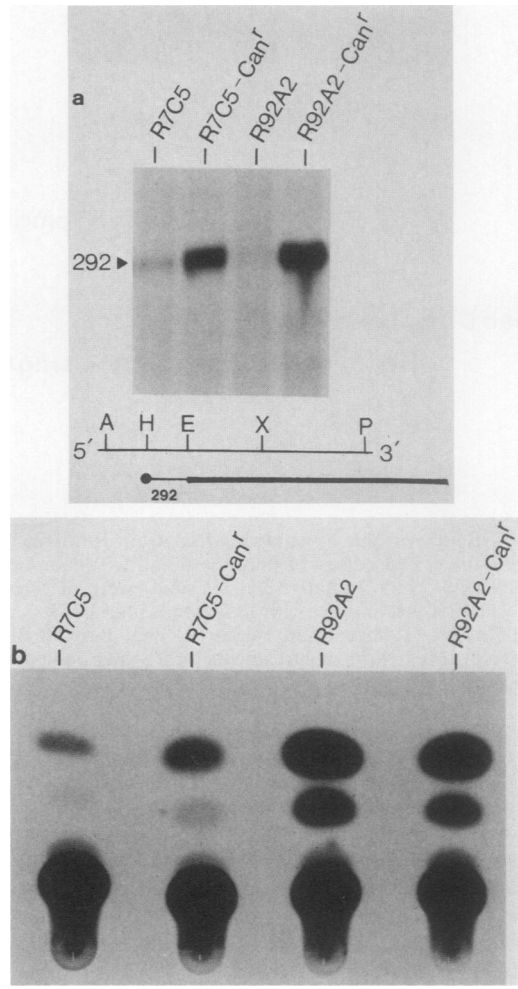

FIG. 7. Lack of *trans* induction in stable cell lines. Cloned RPMI 2650 cell lines containing pmgAS-9 and RSVneo were placed in DMEM−a+c containing 3 μM canavanine. Two weeks later canavanine-resistant variants were cloned, propagated, and assayed for expression of both CAT and of the endogenous argininosuccinate synthetase mRNA. (a) S1 nuclease assays of canavanine-resistant variants and parental cell lines. The probe contains a *Hin*dIII-*Eco*RI restriction fragment of the human argininosuccinate synthetase cDNA, pAS419 (5), cloned into pUC-8. The probe was linearized by digestion with *Hin*dIII and 3′ end labeled at the *Hin*dIII sites. The predicted length of the protected fragment is 292 bases as indicated. A 50-μg sample of RNA was used in all assays. The following are restriction sites in the full-length cDNA: A, *Ava*I; H, *Hin*dIII; E, *Eco*RI; X, *Xho*I; P, *Pvu*II. (b) CAT expression in cell lines described above. The assays were performed as described in the legend to Fig. 2.

DISCUSSION

Our data show that the CAT gene under the transcriptional control of the human argininosuccinate synthetase promoter is subject to arginine-mediated repression in RPMI 2650 cells. All of the CAT minigenes contain 88 bp of exon sequence from the human argininosuccinate synthetase gene, and it is possible that these sequences play a role in the arginine-mediated repression. This role could be at the level of gene transcription or mRNA stability. However, as observed with most regulated eucaryotic genes, it is likely that the controlling *cis*-acting sequences are located in the 5′-flanking region. Minigene constructs containing deletions and various mutations will be useful in identifying the *cis*-acting sequences more precisely.

It is important to note that the degree of CAT repression seen in the RPMI 2650 cells is in the range of that observed for the human argininosuccinate synthetase gene. Since pmgAS-10 (containing only 149 bp of 5′-flanking sequence) and pmgAS-9 (containing approximately 3 kb of 5′-flanking sequence) express with equal efficiency (unpublished observations) and regulate to the same degree, all of the *cis*-acting sequences required for efficient expression and arginine-mediated repression are likely to be in the region of the transcription initiation site. This situation is similar to that observed for many other eucaryotic genes that are regulated by hormones (13), metals (3, 13, 29) or tissue-specific *trans*-acting factors (34). In most cases, the *cis*-acting elements lie within 300 bp of the transcription initiation site. It was previously postulated that exon 2 sequences might play a role in the arginine-mediated repression since this exon

contains three tandem arginine codons (1). It was speculated that repression might occur through an attenuation mechanism involving Arg-tRNA (1). Our data demonstrate that this is not likely since none of the CAT minigenes contain exon 2 sequences and all of them are subject to the arginine-mediated repression in RPMI 2650 cells. Furthermore, we have constructed a CAT minigene that contains exon 2 cloned into the *Bam*HI site in the hybrid intron in pmgAS-9 and have found that this construct regulates to the same degree as pmgAS-9 or pmgAS-10 (Freytag et al., unpublished data). Therefore, exon 2 sequences do not appear to play a role in the arginine-mediated repression.

Variants of RPMI 2650 cells which are able to grow in the presence of canavanine, a toxic arginine analog, have markedly altered regulation of argininosuccinate synthetase. These variant cells express their endogenous argininosuccinate synthetase gene at greatly elevated levels, and the high constitutive expression is not due to gene amplification (31). Thus, these cells presumably avoid canavanine toxicity by increased synthesis of arginine. The canavanine-resistant variants do not show arginine-mediated repression of the endogenous argininosuccinate synthetase gene or the CAT minigenes. It is possible that a single event, such as the loss of a negative-acting regulator, results in the increased expression (derepression) and simultaneous loss of arginine-mediated repression. This hypothesis implies that the factor mediating the arginine repression is identical to that responsible for the high constitutive expression.

Expression of the CAT minigenes in RPMI 2650 cells and Can^r-1 cells suggests that arginine-mediated repression is independent of the mechanism causing canavanine resistance. We introduced the CAT minigenes into both RPMI 2650 cells and canavanine-resistant variants of this cell line and assayed for transient expression of CAT. The results of these experiments showed that the expression of CAT was equal in both cell types. It was possible that the amount of DNA used in the transfections was sufficient to titrate out a necessary cellular *trans*-acting factor and thereby suppress the *trans* induction. Therefore, we repeated these experiments using CAT minigenes containing the SV40 enhancer which allowed us to reduce the amount of DNA used in the transfection 200- to 500-fold and still obtain measurable CAT activity. The results of these experiments showed no difference in CAT expression between RPMI 2650 cells and the Can^r-1 cells. Therefore, the *trans* induction could not be reproduced in a transient expression assay with a CAT minigene having approximately 3 kb of 5′-flanking sequence. We next tested for the *trans* induction using cell lines containing integrated copies of the CAT minigenes. We preselected cell lines having five copies or fewer to eliminate the possibility of titrating out cellular factors that interact specifically with argininosuccinate synthetase promoter sequences. When canavanine-resistant variants were established and assayed for CAT activity and argininosuccinate synthetase mRNA levels, it was shown that the endogenous gene was expressed at greatly elevated levels, whereas CAT expression was relatively unchanged. Since these cell lines do exhibit arginine-mediated repression of CAT (Fig. 2, data not shown), we conclude that the arginine-mediated repression and *trans* induction are separate regulatory events.

There are several models which explain why canavanine-resistant cells lose arginine-mediated repression. For example, a strong positive-acting mechanism could dominate over the arginine-mediated repression. Expression of the argininosuccinate synthetase gene in somatic cell hybrids made between RPMI 2650 cells and Can^r cells has strongly indicated that the *trans* induction occurs through a positive-acting mechanism (F. Boyce and S. Freytag, unpublished data). Alternatively, canavanine-resistant cells may synthesize enough arginine to become indifferent to the extracellular arginine concentration.

We were unable to observe significant *trans* induction using the CAT minigenes. It is possible that the *cis*-acting sequences which mediate this regulation occur further upstream of the flanking region used in our constructs or lie within the gene itself. Alternatively, the *trans* induction in canavanine-resistant cells may be regulated at the posttranscriptional level. Thus, the mechanism involved in the *trans* induction appears to be quite different from that responsible for the arginine-mediated repression. It will be interesting to elucidate the molecular details of these two regulatory systems and how they interact to control the expression of a human gene.

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LITERATURE CITED

1. **Bock, H., T. Su, W. O'Brien, and A. Beaudet.** 1983. Sequence for human argininosuccinate synthetase cDNA. Nucleic Acids Res. **11**:6505–6512.
2. **Breathnach, R., and P. Chambon.** 1981. Organization and expression of eucaryotic split genes coding for proteins. Annu. Rev. Biochem. **50**:349–383.
3. **Carter, A., B. Felber, M. Walling, M. Jubier, C. Schmidt, and D. Hamer.** 1984. Duplicated heavy meal control sequences of the mouse metallothionein-I gene. Proc. Natl. Acad. Sci. USA **81**:7392–7396.
4. **Dynan, W., and R. Tjian.** 1983. The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. Cell **35**:79–87.
5. **Freytag, S., A. Beaudet, H. Bock, and W. O'Brien.** 1984. Molecular structure of the human argininosuccinate synthetase gene: occurrence of alternative mRNA splicing. Mol. Cell. Biol. **4**:1978–1984.
6. **Freytag, S., and K. Collier.** 1984. Molecular cloning of a cDNA for human pyruvate carboxylase: structural relationship to other biotin-containing carboxylases and regulation of mRNA content in differentiating preadipocytes. J. Biol. Chem. **259**: 12831–12837.
7. **Gorman, C., L. Moffat, and B. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
8. **Gorman, C., P. Padmanabhan, and B. Howard.** 1983. High efficiency DNA transformation of primate cells. Science **221**:551–553.
9. **Hudson, L., R. Erbe, and L. Jacoby.** 1980. Expression of the human argininosuccinate synthetase gene in hamster transferents. Proc. Natl. Acad. Sci. USA **77**:4234–4238.
10. **Imperiale, M., L. Feldman, and J. Nevins.** 1983. Activation of gene expression by adenovirus and herpesvirus regulatory genes acting in trans and by a cis-acting adenovirus enhancer element. Cell **35**:127–136.
11. **Irr, J., and L. Jacoby.** 1978. Control of argininosuccinate synthetase by arginine in human lymphoblasts. Somatic Cell Genet. **4**:111–124.
12. **Jacoby, L.** 1978. Canavanine-resistant variants of human lymphoblasts. Somatic Cell Genet. **4**:221–231.
13. **Karin, M., A. Haslinger, H. Holtgreve, R. Richards, P. Krauter,**

H. Westphal, and M. Beato. 1984. Characterization of DNA sequences through which cadmium and glucocorticoid hormones induce human metallothionein-IIA gene. Nature (London) **308**:513–519.
14. **Luskey, K., J. Faust, D. Chin, M. Brown, and J. Goldstein.** 1983. Amplification of the gene for 3-hydroxy-3-methylglutaryl coenzyme A reductase, but not for the 53-kDa protein, in UT-1 cells. J. Biol. Chem. **258**:8462–8469.
15. **Maniatis, T., E. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **McKnight, G., and R. Palmiter.** 1979. Transcriptional regulation of the ovalbumin and conalbumin genes by steroid hormones in chick oviduct. J. Biol. Chem. **254**:9050–9058.
17. **Mercola, M., J. Goverman, C. Mirell, and K. Calame.** 1985. Immunoglobulin heavy-chain enhancer requires one or more tissue-specific factors. Science **227**:266–270.
18. **Myers, R., D. Rio, A. Robbins, and R. Tjian.** 1981. SV40 gene expression is modulated by the cooperative binding of T antigen to DNA. Cell **25**:373–384.
19. **Parker, B., and G. Stark.** 1979. Regulation of SV40 transcription: sensitive analysis of the RNA species present early in infection by virus or virus DNA. J. Virol. **31**:360–369.
20. **Payvar, F., D. DeFranco, G. Firestone, B. Edgar, O. Wrange, S. Okret, J. Gustafsson, and K. Yamamoto.** 1983. Sequence-specific binding of glucocorticoid receptor to MTV DNA at sites within and upstream of the transcribed region. Cell **35**:381–392.
21. **Payvar, F., O. Wrange, J. Carlstedt-Duke, S. Okret, J. Gustafsson, and K. Yamamoto.** 1981. Purified glucocorticoid receptors bind selectively *in vitro* to a cloned DNA fragment whose transcription is regulated by glucocorticoids *in vivo*. Proc. Natl. Acad. Sci. USA **78**:6628–6632.
22. **Pelham, H.** 1982. A regulatory upstream promoter element in the *Drosophila* hsp70 heat-shock gene. Cell **30**:517–528.
23. **Reddy, V., B. Thimmappaya, R. Dhar, K. Subramanian, B. Zain, J. Pan, P. Ghosh, M. Celma, and S. Weissman.** 1978. The genome of simian virus 40. Science **200**:494–527.
24. **Reynolds, G., S. Basu, T. Osborne, D. Chin, G. Gil, M. Brown, J. Goldstein, and K. Luskey.** 1984. HMG CoA reductase: a negatively regulated gene with unusual promoter and 5′ untranslated regions. Cell **38**:275–285.
25. **Richards, R., A. Heguy, and M. Karin.** 1984. Structural and functional analysis of the human metallothionein-IA gene: differential induction by metal ions and glucocorticoids. Cell **37**:263–272.
26. **Ringold, G., K. Yamamoto, J. Bishop, and H. Varmus.** 1977. Glucocorticoid-stimulated accumulation of mouse mammary tumor virus RNA: increased rate of synthesis of viral RNA. Proc. Natl. Acad. Sci. USA **74**:2879–2883.
27. **Schimke, R.** 1964. Enzymes of arginine metabolism in mammalian cell culture repression of argininosuccinate synthetase and argininosuccinase. J. Biol. Chem. **239**:136–145.
28. **Sequin, C., B. Felber, A. Carter, and D. Hamer.** 1984. Competition for cellular factors that activate metallothionein gene transcription. Nature (London) **312**:781–785.
29. **Stuart, G., P. Searle, H. Chen, R. Brinster, and R. Palmiter.** 1984. A 12-base-pair DNA motif that is repeated several times in metallothionein gene promoters confers metal regulation to a heterologous gene. Proc. Natl. Acad. Sci. USA **81**:7318–7322.
30. **Su, T., A. Beaudet, and W. O'Brien.** 1981. Increased translatable mRNA for argininosuccinate synthetase in canavanine-resistant human cells. Biochemistry **20**:2956–2960.
31. **Su, T., H. Bock, W. O'Brien, and A. Beaudet.** 1981. Cloning of cDNA for argininosuccinate synthetase mRNA and study of enzyme overproduction in a human cell line. J. Biol. Chem. **256**:11826–11831.
32. **Sutcliffe, J.** 1978. Complete nucleotide sequence of *E. coli* plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. **43**:77–90.
33. **Tooze, J.** 1981. DNA tumor viruses: molecular biology of tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Walker, M., T. Edlund, A. Boulet, and W. Rutter.** 1983. Cell-specific expression controlled by the 5′-flanking region of insulin and chymotrypsin genes. Nature (London) **306**:557–561.