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Both Upstream and Intron Sequence Elements Are Required for Elevated Expression of the Rat Somatic Cytochrome *c* Gene in COS-1 Cells

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To investigate the transcriptional control of nuclear-encoded respiratory genes in mammals, we have performed a deletional analysis of *cis*-acting regulatory sequences in the rat somatic cytochrome *c* gene. Three major regions are required for maximal expression of the transfected gene in kidney cell lines CV-1 and COS-1. One of these, region III (+71 to +115 from the transcription initiation site), is an unusual intragenic controlling element found in the 5′ end of the first intron, while the other two, region I (−191 to −165) and region II (−139 to −84), define the upstream promoter. Region II contains two consensus CCAAT boxes and mediates a constitutive level of expression in both cell lines. In contrast, regions I and III are both required for the increased promoter activity observed in COS-1 cells compared with promoter activity observed in CV-1 cells, and the regions function individually as competitors with the full promoter for *trans*-acting factors or complexes. Region III contains a perfect octanucleotide homology with region I in addition to a consensus Sp1-transcription-factor-binding site. Promoter stimulation in COS-1 cells can be duplicated in CV-1 cells by cotransfecting with a T-antigen-producing vector, but purified T antigen does not bind anywhere in the cytochrome *c* promoter. A control promoter from the mouse metallothionein I gene is similarly activated in T-antigen-producing cells only in the presence of zinc, which activates its upstream regulatory sites. We conclude that T antigen stimulates these cellular promoters through the activation or induction of cellular factors or complexes that mediate their effects through promoter-specific regulatory elements. Cytochrome *c* promoter regions activated in this system may play a physiological role in controlling gene expression.

In mammals, the mitochondrial oxidative complexes consist of a minimum of 67 different polypeptides with all but 13 encoded in the nuclear genome (reviewed in references 2 and 13). Although the polypeptides specified by the mitochondrial genome are identical in all tissues, many nuclear-encoded subunits have tissue-specific genetic variants (8, 22), suggesting that over 100 nuclear genes encode the structural and catalytic components of the mitochondrial oxidative phosphorylation system. The proteins of the respiratory chain are coordinately regulated over a 10-fold range according to tissue-specific energy demands (28, 41) and are subject to thyroid hormone control as well (4, 7, 45). Moreover, transformation by viral and cellular oncogenes can increase mitochondrial gene expression (9). Although variation of the number of mitochondrial genomes per cell is thought to be a major regulatory mechanism for the expression of the mitochondrial-encoded subunits (30), nothing is known about the molecular mechanisms governing either the tissue-specific or the hormonal control of the large family of nuclear-encoded respiratory genes.

Cytochrome *c* is the product of nuclear genes, resides in the intermembrane space, and functions in the catalytic transfer of electrons between respiratory complexes III and IV (reviewed in references 29 and 40). One mammalian isoform of cytochrome *c* is expressed in somatic tissues, and a second is expressed during spermatogenic differentiation (14). The rat somatic cytochrome *c* gene has introns dividing its 5′ noncoding and coding regions and directs the synthesis of 1,400-, 1,100-, and 700-nucleotide mRNAs (32, 36). These mRNAs are coordinately expressed in somatic tissues and differ in the lengths of their 3′ noncoding regions. Each mRNA serves as a template for the numerous processed cytochrome *c* pseudogenes found in the genomes of rats and other mammals (32, 34). The mRNA levels parallel tissue-specific variations in respiratory proteins, suggesting pretranslational control of cytochrome *c* expression (36). In addition, thyroid hormone modulates the amount of cytochrome *c* mRNA in rat liver and kidney at least in part through a transcriptional mechanism (35). In this paper, we describe multiple *cis*-acting sequence elements that contribute to gene activity in kidney cell lines. Two of these elements, one upstream and the other within the intervening sequence, are specifically *trans*-activated in COS-1 cells relative to the parental cell line CV-1 and are thus distinguished from the CCAAT box core promoter.

MATERIALS AND METHODS

Construction of plasmids. The parent vector pRC4CAT, used for the deletion analysis of cytochrome *c* promoter function, was constructed by cloning a 3.2-kilobase *Eco*RI-*Hin*dIII fragment containing the rat cytochrome *c* gene and its flanking DNA (33) into the *Eco*RI-*Sal*I fragment of pSV0d (27) with *Xho*I and *Kpn*I linkers (Fig. 1). The 380-base-pair (bp) *Dra*I-*Acc*I cytochrome *c*-coding region was replaced with the 716-bp *Hin*dIII-*Ban*I chloramphenicol acetyltransferase (CAT)-coding region from pSV2-CAT (10) by using *Bgl*II linkers. The initial promoter deletions were made by digesting pRC4CAT with the restriction enzymes indicated in Fig. 3, treating with either Klenow fragment or T4 DNA polymerase to produce blunt ends, and recircularizing with *Xho*I linkers. Additional deletions were made with *Bal* 31 followed by T4 DNA polymerase treatment and *Xho*I linker

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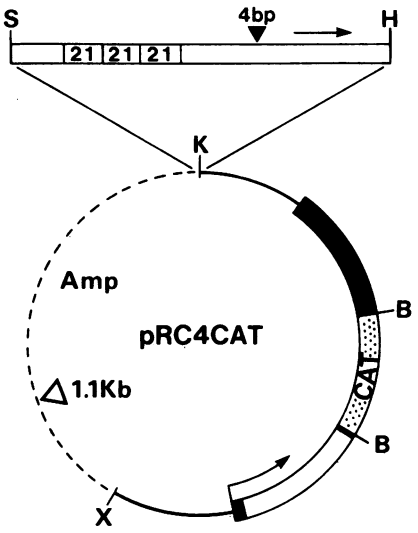

FIG. 1. Structures of vector pRC4CAT and its replicating derivative pRC4CATori. In pRC4CAT, the coding region and small intron of rat somatic cytochrome *c* gene were replaced with the CAT-coding region (▨). The remainder of the gene, including its large intron (▭), 5′ and 3′ noncoding regions (▬), and flanking DNA (——) was unchanged. The 1.1 kilobase (kb) of DNA that inhibits replication in mammalian cells was deleted from the vector sequences from pBR322 (-----). The replicating vector pRC4CATori contained the enhancerless *Sph*I-*Hin*dIII SV40 origin fragment cloned in the indicated orientation into the *Kpn*I site at the 3′ end of the cytochrome *c* gene. For some experiments, the SV40 origin was replaced with a replication-defective origin, designated oriΔ, that had a 4-bp deletion at the *Bgl*I site in the origin. Arrows indicate the direction of transcription from cytochrome *c* and SV40 early promoters. B, *Bgl*II; X, *Xho*I; S, *Sph*I; H, *Hin*dIII; K, *Kpn*I.

addition. The extent of each *Bal* 31-generated deletion was verified by sequencing. For use as a control promoter, the *Eco*RI-*Bgl*II mouse metallothionein I promoter fragment (−1500 to +69) from pMT-TK (26) replaced the *Xho*I-*Rsr*II cytochrome *c* promoter (−726 to +56) of pRC4CAT by use of *Xho*I and *Bgl*II linkers. The replicating vector pRC4CATori contains the 200-bp *Sph*I-*Hin*dIII simian virus 40 (SV40) origin fragment (SV40 nucleotides 5171 to 130) cloned into the *Kpn*I site of pRC4CAT with *Kpn*I linkers (Fig. 1). It was converted to the replication-defective derivative pRC4CAToriΔ by substitution of the 4-bp origin deletion from pLP11-CAT (12).

DNA transfections and CAT assays. CV-1 and COS-1 cells were replated 24 h before transfection. The cells were transfected with 30 μg of plasmid DNA by the calcium phosphate precipitate method (11) followed by a 15% glycerol shock 4 h later. The cells from one-half of each plate were harvested for assay of CAT activity 48 h after glycerol shock, with [^{14}C]chloramphenicol as the substrate (10). Radioactive chloramphenicol and its acetylated derivatives were resolved chromatographically and quantified by scintillation counting. To minimize variability resulting from differences in transfection frequency, all CAT values were normalized to the amount of extrachromosomal CAT DNA recovered from each plate. Hirt supernatants were therefore prepared from the remaining cells (15), and the amount of CAT DNA was determined by slot blot hybridization and densitometry with a pRC4CAT standard curve for comparison. Final values for each construct were the average of between 4 and 10 determinations and were expressed as milliunits of CAT per nanogram of CAT DNA (1 unit = 1 nmol/min at 30°C). The mouse metallothionein I promoter was stimulated by the addition of 100 μM zinc sulfate to the medium 24 h after glycerol shock (38). Values were adjusted for variation between different batches of cells with pRC4CAT as a control.

DNA sequence analysis. Sequences were determined by the dideoxy-chain-termination method (31) or the chemical-degradation method (25).

T-antigen binding. T-antigen-binding reactions were performed in 20 μl of 20 mM PIPES (piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]; pH 7.0)–50 mM NaCl–1 mM dithiothreitol–0.1 mM EDTA–10% (vol/vol) glycerol containing 0.5 ng of end-labeled fragment, 100 ng of sonicated calf thymus DNA, and 7 ng of immune-purified T antigen (B. Stillman, Cold Spring Harbor Laboratories). Reaction products were analyzed by electrophoresis as described by Strauss and Varshavsky (42).

RESULTS

Activation of rat somatic cytochrome *c* promoter in COS-1 cells. To begin to define promoter elements responsible for the expression of the rat somatic cytochrome *c* gene RC4 (33), pRC4CAT was constructed as the starting vector for the analysis of deletion mutants (Fig. 1). This vector preserves the integrity of the gene and its flanking DNA. Only the cytochrome *c*-coding region, including its 105-bp intron, is replaced with the CAT-coding sequence for assay of promoter activity (10). Upon transfection, pRC4CAT produced sevenfold more CAT activity per gene copy in COS-1 cells than in the parent cell line CV-1 (Fig. 2, −726). The transcription initiation sites observed in COS-1 and CV-1 cells were identical to that defined in rat liver by S1 nuclease mapping, and the CAT activities accurately reflected the levels of properly initiated mRNAs (data not shown). In contrast, when the cytochrome *c* promoter was replaced by the mouse metallothionein I promoter as a control, the level of CAT activity was identical in the two cell lines. However, in the presence of zinc, the metallothionein promoter was severalfold more active in COS-1 cells than in CV-1 cells and was comparable in strength to the intact cytochrome *c* promoter (Fig. 2). Zinc induces the metallothionein promoter through well-characterized upstream regulatory elements (43) that bind stimulatory *trans*-acting transcription factors (39). Therefore, promoter-specific sites may mediate the elevated CAT activity observed in COS-1 cells.

Expression of rat cytochrome *c* promoter and its activation in COS-1 cells modulated by distinct sequence elements. To define the putative sequence elements required for cytochrome *c* promoter activation, an initial series of 5′ deletions was assayed for CAT expression to determine the ratio of promoter function in COS-1 relative to that in CV-1 (Fig. 2). All promoters with sequences upstream from nucleotide position −215 from the cap site were clearly enhanced in COS-1 cells, whereas those containing only sequences downstream from position −159 were not. In addition, small internal deletions (−207 to −159 and −191 to −159) significantly diminished promoter activation (Fig. 2) and thus further substantiated the significance of this region. Adjacent sequences, defined by an internal deletion between −165 and −139, did not modulate the strength of the promoter or its stimulation in COS-1 cells. Therefore, the sequence between nucleotides −191 and −165 (designated promoter region I) generally contributed to the strength of the cytochrome *c* promoter and was required for its stimulation in COS-1 cells.

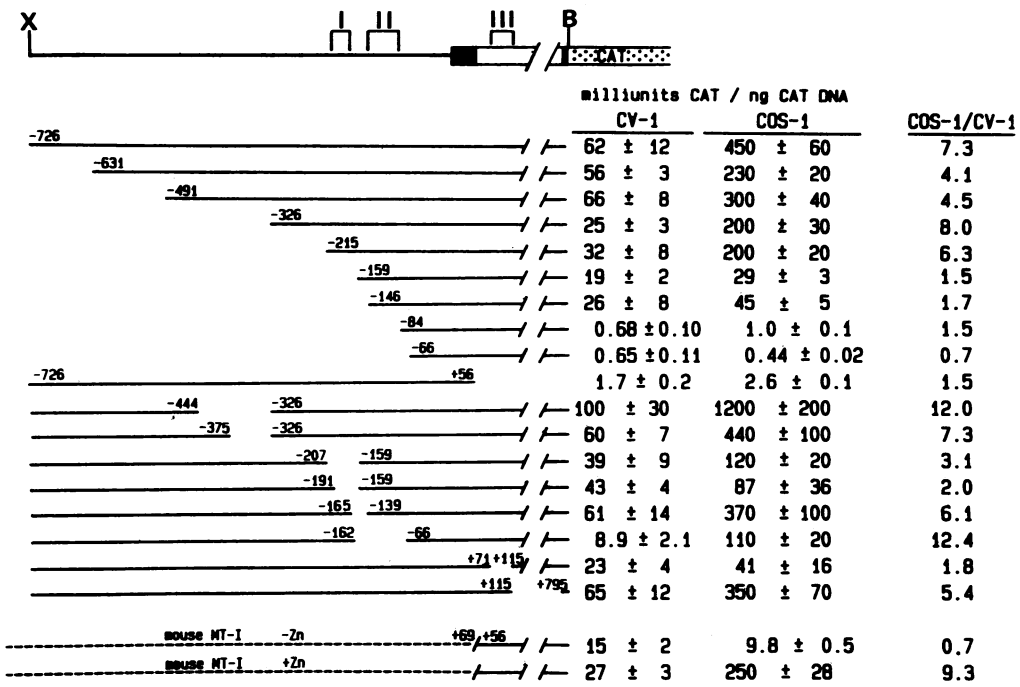

FIG. 2. Deletional analysis of cytochrome *c*-CAT chimeric genes. The top line depicts a portion of the map of pRC4CAT, and the deletions in this region are shown below. The activity of each deletion with the indicated endpoints is expressed as milliunits of CAT activity per nanogram of CAT DNA recovered in Hirt supernatants (± standard error of the mean) to control for transfection efficiency. The level of activity in COS-1 cells relative to activity in CV-1 cells is shown in column 3. The mouse metallothionein I (MT-I) fusion contained the mouse metallothionein I promoter from −1500 to +69 joined to the cytochrome *c* gene at +56 and was assayed in the absence (−Zn) or presence (+Zn) of 100 μM zinc added 24 h posttransfection. I, II, and III, Major promoter elements defined by the deletions; B, *Bgl*II; X, *Xho*I.

In contrast to results with region I, removal of a second distinct sequence element (designated promoter region II), bounded by 5′ deletions at −146 and −84, resulted in a quantitatively similar decrease in both cell lines. An internal deletion from −162 to −66 that spans region II diminished promoter strength in both cell lines but did not diminish activation of the promoter in COS-1 cells. Region II can be further localized to between −139 and −84, because the deletion of sequences from −165 to −139 did not diminish promoter activity. The presence of consensus CCAAT boxes in region II (Fig. 3) was consistent with the function of this region as a constitutive core promoter required for basal-level expression. The 5′ deletions also suggested the presence of a second constitutive promoter sequence located between −491 and −326. The removal of this region consistently produced a twofold drop in promoter activity in the 5′ deletion series (Fig. 2 and Table 1). Although this region contained two possible Sp1-binding sites, two internal deletions (−444 to −326 and −375 to −326) demonstrated that these sites did not stimulate cytochrome *c* promoter activity. This upstream region has not been defined further.

The deletion of cytochrome *c* sequences between +56 and the *Bgl*II linker (Fig. 2) removed the large intron located between positions +61 and +856 and markedly reduced CAT activity in both cell lines. Surprisingly, promoter stimulation in COS-1 cells was also lost in this construct despite the presence of the intact upstream region I. A large deletion within the intron (between +115 and +795) had no effect on CAT activity. In contrast, a small deletion in the 5′ end of the intron (between +71 and +115), which left the donor splice junction intact, had a moderate effect on CAT activity in CV-1 cells but mediated a more drastic decrease in COS-1 cells, thereby abolishing the promoter activation observed in these cells. This 44-bp intron region (designated region III) contained a consensus Sp1-binding site (GGGGCGGGA) adjacent to an octanucleotide sequence (AGAGGGCG) also found in an inverted orientation in upstream region I (Fig. 3). Thus, the increased cytochrome *c* promoter activity in COS-1 cells required the presence of two distinct elements. One was located in the 5′ upstream region, and the other was located within the first intron. Neither region alone was sufficient to permit the stimulation of the cytochrome *c* promoter.

***trans*-Activation of specific promoter sites in COS-1 cells.** If *trans*-acting factors stimulate the promoter in COS-1 cells through their interaction with the specific promoter sites defined by deletion analysis, it should be possible to titrate the factors away in competition experiments (39). This was

TABLE 1. Deletion analysis of the rat cytochrome *c* promoter with replicating vectors in COS-1 cells[a]

Deletion	Promoter activity (mU of CAT/ng of CAT DNA)[b]
None	1,100 ± 200
−631	930 ± 140
−491	1,200 ± 300
−326	510 ± 70
−215	550 ± 110
−159	290 ± 40
−66	29 ± 2
+71 to +115	350 ± 50

[a] COS-1 cells were transfected as shown in Fig. 2, with a series of 5′ deletions of the cytochrome *c* promoter on vectors containing the SV40 origin of replication. Each construction was assayed a minimum of four times.
[b] Data are expressed as ± standard error of the mean.

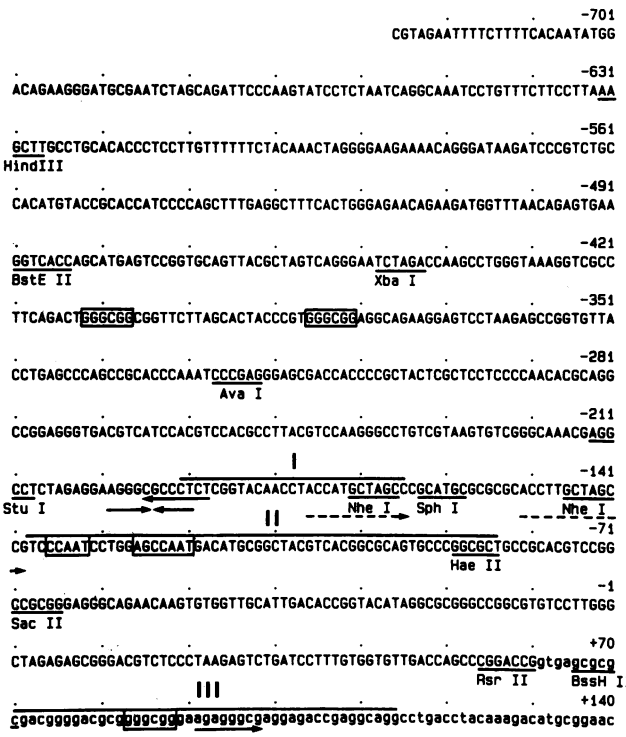

FIG. 3. Sequence of the rat somatic cytochrome *c* promoter. Intron sequences are shown in lowercase letters. Regions I, II, and III are overlined, and the CCAAT and GGGCGG sequences are boxed. The solid arrows underline an octanucleotide found in promoter regions I and III; the broken arrows underline a 12-bp sequence directly repeated in the upstream region. Restriction sites used for construction of deletions are shown below the sequence.

accomplished either by cotransfecting an excess of these promoter regions and assessing their effect on the intact promoter (Fig. 4) or by determining the relative activity of deletion mutants after elevation of their template copy number by vector replication (Table 1). When the cytochrome *c* upstream region between −726 and −162 (cloned into pBR322) was used as a competitor, the CAT activity directed by the intact cytochrome *c* promoter was significantly reduced at a competitor-to-reference-plasmid ratio of 29:1, whereas the CAT activity directed by the adenovirus EII promoter of pEIICAT remained unaffected. Failure of the EII promoter to be influenced by cytochrome *c* promoter sequences in *trans* reaffirmed that stimulation occurred through promoter-specific sites. A competitor plasmid containing only cytochrome *c* upstream sequences between −209 and −162 was identical to the larger upstream region in diminishing pRC4CAT activity, which demonstrated the specific interaction of limiting factors with region I. A marked 10-fold reduction in pRC4CAT activity was achieved at a lower ratio of 15:1 with a competitor plasmid containing sequences from intron region III between +60 and +115 and confirmed that the *cis*-acting intron effects were mediated at the DNA level and were not posttranscriptional. The more-efficient competition of region III was in keeping with the 10-fold reduction in activity caused by its deletion and suggested that regions I and III bind different factors or bind the same factor with different affinities.

If the interaction of limiting *trans*-acting factors with specific sequence elements is required for enhanced promoter activity per gene copy, an excess of promoter templates should decrease the dependence of the promoter on *trans*-activated elements, because a smaller fraction of promoters will be stimulated through these elements. The deletion of these elements should therefore have a smaller effect on CAT activity per template on high-copy vectors compared with that on lower-copy vectors. Plasmids containing an SV40 origin of replication are replicated to a high copy number in COS-1 cells (27). The 200-bp *Hin*dIII-*Sph*I SV40 origin fragment, lacking the 72-bp enhancers, was therefore introduced into a series of deletion derivatives of pRC4CAT (Fig. 1) to assay the effect of each derivative when replicated to high copy. Replication increased CAT vector DNA levels in Hirt supernatants approximately 20-fold over nonreplicating vector levels, and S1 nuclease analysis verified the utilization of identical transcriptional start sites on replicating and nonreplicating vectors (data not shown). Table 1 shows only a modest decrease in CAT activity upon removal of promoter region I (between −215 and −159) and intron region III (between +71 and +115) from replicating vectors in COS-1 cells. This decrease contrasts with the 7- to 10-fold reduction in promoter activity observed with the same deletions in the absence of replication and is comparable to the relative effects of the deletions in CV-1 cells (Fig. 2). Similarly, the importance of CCAAT-box-containing region II was reduced from 40-fold on nonreplicating vectors to about 10-fold on replicating vectors. Thus, the effects of an excess of specific promoter sites, introduced either by increasing the number of promoter templates or by cotransfecting competitor plasmids containing these sites, were consistent with the activation of the sites through *trans*-acting factors or complexes.

Role of T antigen in cytochrome *c* promoter activation. Despite reduced dependence on cytochrome *c* promoter elements, the replicating vectors in COS-1 cells produced approximately 15- to 20-fold-higher levels of CAT activity per gene copy than nonreplicating vectors in CV-1 cells did. Because supercoiling may increase gene transcription (re-

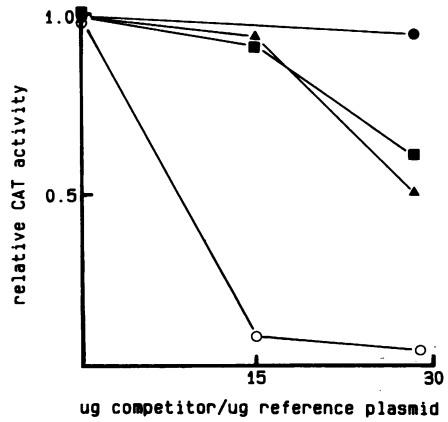

FIG. 4. Competition of cytochrome *c* promoter fragments. Reference plasmids (1 μg) were cotransfected with 0, 15, or 29 μg of competitor plasmids containing cytochrome *c* promoter sequences cloned into pBR322. pEII CAT has the CAT gene under the control of the 300-bp adenovirus EII promoter, and its competitor plasmid has the full cytochrome *c* upstream promoter −726 to −162 (●). Competitor plasmids for pRC4CAT activity include cytochrome *c* promoter fragments −726 to −162 (■), −209 to −162 (▲), and +60 to +115 (○). For each transfection, the total DNA was adjusted to 30 μg with pBR322. CAT activities were normalized to the amount of CAT DNA recovered in Hirt supernatants and are expressed as relative activity in the absence and presence of competitor.

viewed in reference 46), the stimulatory effect of the replication origin may be mediated indirectly through plasmid supercoiling resulting from T-antigen-dependent replication. Alternatively, in pRC4CATori the cytochrome *c* promoter is positioned 2.5 kilobases downstream from the late side of the SV40 origin, and T antigen may *trans*-activate this promoter, as it does the SV40 late genes, through specific origin sites (5, 6, 18, 19). To distinguish between these T-antigen-dependent activities, a replication-defective origin, identical to that in pRC4CATori except for a 4-bp deletion in T-antigen-binding site II (12), was used for the construction of pRC4CAToriΔ, a replication-defective derivative. The effects of T antigen on both the full cytochrome *c* promoter and the −159 deletion were then monitored in vectors containing no origin, the replication-defective origin, or the intact origin upon cotransfection of each vector into CV-1 cells with pRSV-T (24), which directs the synthesis of T antigen (Table 2).

The full cytochrome *c* promoter, but not the −159 deletion, was stimulated severalfold by T antigen under conditions in which the activity of the adenovirus EII promoter, a positive control, was substantially increased (Table 2), as previously described (24). Similarly, the mouse metallothionein I promoter was stimulated by T antigen in the presence but not the absence of zinc. Cotransfection with T antigen resulted in a somewhat lower elevation of both cellular promoters compared with the level observed in COS-1 cells. Nevertheless, the similar dependence of the cellular promoters on upstream sequences in this experiment supports our previous conclusion that activation is mediated through promoter-specific sites. The presence of the SV40 origin alone did not stimulate the cytochrome *c* promoter in the absence of T antigen (compare pRC4CAT and pRC4CAT ori), and the origin-mediated stimulation of the cytochrome *c* promoter by T antigen was quantitatively the same regardless of whether the origin was competent for replication (compare ori and oriΔ constructs). The failure of oriΔ vectors to replicate was verified by *Dpn*I and *Mbo*I digestion of extrachromosomal DNA (data not shown). Thus, the stimulation of origin-containing vectors by T antigen did not require replication-dependent supercoiling of the vector.

TABLE 2. Effects of T antigen and vector replication on cytochrome *c* promoter activity in CV-1 cells[a]

Promoter vector	Promoter activity (mU of CAT/ng of CAT DNA) for plasmid:		Promoter activity ratio, pRSV-T/pBR322
	pBR322	pRSV-T	
pEIICAT	0.08 ± 0.02	4.8 ± 0.8	60
pRC4CAT	33 ± 8	84 ± 9	2.6
pRC4CAToriΔ	33 ± 2	180 ± 20	5.5
pRC4CATori	43 ± 7	360 ± 40	8.2
pRC4CAT/−159	18 ± 2	21 ± 2	1.2
pRC4CAToriΔ/−159	14 ± 2	130 ± 12	9.1
pRC4CATori/−159	15 ± 3	150 ± 24	10.0
pMET/RC4CAT −Zn	6.8 ± 0.8	9.9 ± 0.8	1.5
pMET/RC4CAT +Zn	15 ± 1	51 ± 2	3.4

[a] CV-1 cells were cotransfected with 15 μg of CAT plasmids and 15 μg of either pBR322 or pRSV-T. pEIICAT contained CAT under the direction of the adenovirus EII promoter. Derivatives of pRC4CAT included ori vectors with functional SV40 origins and the replication-defective oriΔ containing a 4-bp deletion (Fig. 1). Vectors with the cytochrome *c* promoter deleted to nucleotide −159 are indicated. pMET/RC4CAT had the mouse metallothionein I promoter (−1500 to +69) fused to position +56 in the cytochrome *c* gene (Fig. 2) and was assayed in the absence (−Zn) or presence (+Zn) of 100 μM zinc added 24 h after transfection. All data are expressed as ± standard error of the mean from a minimum of four experiments.

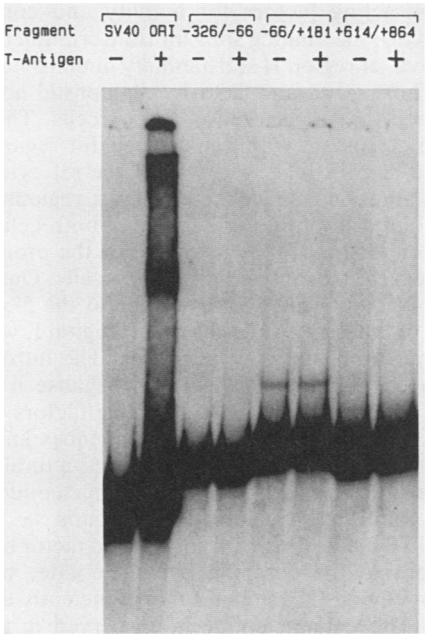

FIG. 5. Binding of T antigen to the cytochrome *c* promoter regions. Fragments (0.5 ng), ^{32}P-end-labeled and containing the SV40 origin region or the indicated regions of the cytochrome *c* gene, were incubated for 30 min at room temperature in the absence (−) or presence (+) of 7 ng of immune-purified T antigen. Reaction mixtures were electrophoresed on a mobility shift gel to detect the formation of protein-DNA complexes.

One possibility is that T antigen activates cellular promoters by directly interacting with binding sites similar to those present on the SV40 replication origin. Binding to these sites may then facilitate the activity of promoter-specific *trans*-regulatory factors or complexes. Sequences resembling T-antigen-binding sites were present in cytochrome *c* promoter region I (Fig. 3, GAGGC at −214 and inverted at −207) as well as in intron region III (AGAGGGCGAGGAGACC at +91 homologous to SV40 T-antigen-binding site II AGAGGCCGAGGCGGCC; GAGGC at +107). Promoter fragments including these sequences (−326 to −66 and −66 to +181) were therefore tested for direct binding of pure T antigen and compared with the SV40 origin fragment from pRC4CATori as well as with an intron fragment (+614 to +864) not required for promoter activation. Incubation of purified T antigen with the SV40 origin fragment containing three T-antigen-binding sites resulted in the formation of a stable protein-DNA complex with decreased electrophoretic mobility (Fig. 5). In contrast, no T-antigen-dependent complexes were detected with any of the cytochrome *c* gene fragments, indicating that T antigen alone does not directly interact with promoter sites.

DISCUSSION

The hormonal regulation of tissue-specific levels of cytochrome *c* mRNAs in rat can occur in part through a transcriptional mechanism (35). In this study, the analysis of a series of deletion mutants in the rat cytochrome *c* gene demonstrated that multiple promoter elements are required for maximal expression. The core promoter region II re-

quires *trans*-acting factors for activity and contains two CCAAT boxes, the binding sites for transcription factor CTF (16). Removal of region II substantially diminished promoter activity in both CV-1 and COS-1 cells but did not alter the promoter activation observed in COS-1 cells. These observations are consistent with a major role for region II in the constitutive basal-level expression of the rat cytochrome *c* gene. Two additional sequence elements, regions I and III, also contributed to promoter activity in both cell lines, but unlike region II, both were required for the promoter activation observed in T-antigen-producing cells. One of these, region III, was defined to within 45 bp at the 5′ end of the first intron of the gene, while the other, region I, was located just upstream from the core promoter. The intron element exerted its effects at the DNA level because it competed effectively for *trans*-acting DNA binding factors or binding complexes and was removed from sequences known to be required for intron splicing. The major structural similarity between regions I and III was the octanucleotide AGAGG GCG. In addition, region III contains a homology (GGGGCGGGA) for the Sp1 transcription factor binding site (17) adjacent to the common octanucleotide, which may explain why region III competes more efficiently than region I in *trans*. The octanucleotide is conserved in the human somatic cytochrome *c* first intron, and a highly homologous sequence is located in the human upstream region adjacent to an Sp1-binding-site consensus sequence (unpublished observations). Regulatory elements are commonly found in multiple copies and have been associated with Sp1-binding sites (44). The sequence elements defined in this study may therefore interact cooperatively to regulate cytochrome *c* promoter activity.

The activation of the transfected rat cytochrome *c* promoter in T-antigen-producing COS-1 cells by means of regions I and III suggests that distinct sequences may mediate the stimulatory effects of viral transforming proteins on other cellular promoters (3, 21, 47). The dependence on zinc for metallothionein I promoter stimulation in COS-1 cells is consistent with a requirement for well-defined upstream sequences that are known to be the sites of heavy-metal activation (44). In addition, cotransfection of CV-1 cells with a T-antigen-producing vector stimulated the intact cytochrome *c* promoter and the zinc-activated metallothionein I promoter. It is unlikely that T antigen itself augments these cellular promoters by direct binding, because the pure protein did not bind to the cytochrome *c* promoter under conditions in which the interaction of T antigen with known binding sites in the SV40 origin was clearly detected. Likewise, activation of the SV40 late genes by T antigen can occur in the absence of T-antigen-binding sites (19). Recently, the transcription factor that mediates the zinc-dependent induction of the human metallothionein II promoter has been shown to be required for maximal activity of the SV40 enhancer (37). Similarly, transcription factor AP-1 interacts with the SV40 enhancer and controls the phorbol ester induction of several cellular genes (1, 23). Therefore, cellular regulatory factors are utilized by SV40 promoter elements and may be generally activated by T antigen as a means of regulating viral promoter function. Our results indicate that some of these factors may functionally interact with *cis*-acting elements in the somatic cytochrome *c* promoter.

The presence of the enhancerless SV40 origin in *cis* and T antigen in *trans* also substantially increased cytochrome *c* promoter activity. S1 nuclease mapping demonstrated the utilization of cellular transcription initiation sites for both replicating and nonreplicating vectors. Replication and supercoiling of the vector were not required, because comparable stimulation occurred through a replication-defective origin. Although the cytochrome *c* promoter elements retained their ability to modulate the level of expression on replicating vectors, their importance was diminished. It is notable in this context that the SV40 origin fragment contained tandem copies, with one mismatch, of the octanucleotide sequence common to cytochrome *c* regions I and III and may function to enhance activity. The presence of enhancers on a vector can mask cellular promoter elements (20), which suggests that the diminished importance of cytochrome *c* promoter sequences on replicating vectors may in part be the result of origin-dependent *trans*-activation of the promoter in addition to titration of cellular transcription factors.

In conclusion, we have identified three major sequence elements required for maximal expression of the cytochrome *c* gene, one of which, surprisingly, was located within the first intron. The characterization of the promoters in COS-1 and CV-1 cells has allowed us to define those sequences necessary for *trans*-activation of the promoters by cellular factors induced or activated by T antigen. These *cis*-acting elements are thus likely to play a role in the control of cytochrome *c* gene expression and may generally participate in the tissue-specific and hormonal regulation of nuclear-encoded respiratory genes in mammalian systems.

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