Positive and Negative Transcriptional Elements of the Human Type IV Collagenase Gene

STEVEN M. FRISCH* AND J. HIROSHI MORISAKI

*Departments of Medicine (Dermatology Division) and Biochemistry/Molecular Biophysics,
Washington University School of Medicine, St. Louis, Missouri 63110*

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Proteolysis by type IV collagenase (T4) has been implicated in the process of tumor metastasis. The T4 gene is expressed in fibroblasts, but not in normal epithelial cells, and its expression is specifically repressed by the E1A oncogene of adenovirus. We present an investigation of the transcriptional elements responsible for basal, E1A-repressible, and tissue-specific expression. 5'-Deletion analysis, DNase I footprinting, and gel mobility shift assays revealed a strong, E1A-repressible enhancer element, r2, located about 1,650 bp upstream of the start site. This enhancer bound a protein with binding specificity very similar to that of the transcription factor AP-2. A potent silencer sequence was found 2 to 5 bp downstream of this enhancer. The silencer repressed transcription from either r2 or AP-1 enhancer elements and in the context of either type IV collagenase or thymidine kinase (tk) gene core promoters; enhancerless transcription from the latter core promoter was also repressed. Comprising the silencer were two contiguous, autonomously functioning silencer elements. Negative regulation of T4 transcription by at least two factors was demonstrated. mcf-7 proteins specifically binding both elements were detected by gel mobility shift assays; a protein of ~185 kDa that bound to one of these elements was detected by DNA-protein cross-linking. The silencer repressed transcription, in an r2 enhancer-tk promoter context, much more efficiently in T4-nonproducing cells (mcf-7 or HeLa) than in T4-producing cells (HT1080), suggesting that cell type-specific silencing may contribute to the regulation of this gene.

The role of secreted proteases in morphogenesis and in pathologic processes such as tumor metastasis has become increasingly apparent (4, 8, 12, 13, 17). That cells can respond to various growth factors and oncogenes by secreting proteases suggests that these genes are under a particularly complex regulation. For example, the interstitial collagenase and stromelysin genes are inducible by interleukin-1, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), *v-src*, and *v-H-ras* and repressible by glucocorticoids, transforming growth factor β (TGF- β) retinoic acid (see reference 17 for a review), and E1A (8). Both positive (AP-1 [1]) and negative (TIF [10]) elements have been identified in these gene promoters. In contrast, regulation of the type IV collagenase (T4) gene is not well characterized: it is resistant to growth factor or lymphokine induction (5). However, its expression does seem to be generally cell type restricted, as fibroblasts, but not epithelial cells, synthesize the gene product. In addition, transcription from the T4 promoter is repressible by the E1A protein of adenovirus (8), which may lead to the loss of metastatic capability observed in E1A-expressing human tumor cells (8). This raises the question of how basal transcription, cell type specificity, and sensitivity to E1A are determined in the promoter.

An analysis of the T4 collagenase 5' transcriptional regulatory region is presented. In particular, a strong enhancer sequence that interacts with a protein having binding cross-specificity with AP-2 was found and shown to be E1A sensitive. Second, a potent, cell type-specific silencer, located adjacent to the enhancer, is described.

MATERIALS AND METHODS

Plasmids. Chloramphenicol transacetylase (CAT) constructs containing or lacking thymidine kinase (tk) were

based on pBLCat2 and pBLCat3 (15), respectively. E1A plasmids were as described before (22), except pE1Afs was constructed to frame-shift E1A after only three amino acids (S. M. Frisch, unpublished data). Double-stranded oligonucleotides were cloned into plasmids by standard procedures (2). All plasmids used for transfections were doubly banded on cesium chloride gradients.

Transfections and CAT assays. Transfections and CAT assays were performed as described previously (8).

Cell lines. Cell lines were cultured in Dulbecco modified Eagle medium with 10% fetal calf serum, 2 mM glutamine, 1 $\times$ penicillin-streptomycin (GIBCO), and, in the case of mcf-7 cells, 1 μ M 17- β -estradiol.

Gel mobility shift assays. Binding conditions were generally as follows: 9% glycerol, 45 mM KCl, 1 mM MgCl₂, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.9), 0.2% gelatin, 0.15 mg of poly(dI-dC) (Pharmacia) per ml, 10⁵ cpm of end-labeled oligonucleotide probe, and 5 to 20 μ g of nuclear protein. After 30 min of binding at 0°C, reactions were loaded onto 4.5% polyacrylamide gels (29:1) and electrophoresed at ~10 V/cm. For the competition experiments, a 100-fold excess of unlabeled competitor DNA was preincubated with extract for 10 min prior to probe addition. Competitor sequences were as follows: GATCGATCGGGGCGGGGCGATC (Sp1); ATTT TGGCTTGAAGCCAATATG (NF1); CTAGTGATGAGTC AGCCGGATC (AP-1); GATCGAAGTACCGCCCCGCG GCCCGT (AP-2); CTAGTGGGACTTTCACAGATC (AP-3); GATCCACACCCACCAGACAAGCCTGAAGCTTGTCT GAAGCCCCG (r2).

DNase I footprinting. Binding reactions (similar to above) were in a total volume of 50 μ l; after binding, 10 μ l of a solution containing 10 mM MgCl₂ and 12.5 mM CaCl₂ was added, plus 0.5 to 2 μ l of DNase I (1 U/ μ l; Promega). Samples were digested for 75 s at room temperature, 100 μ l of transcription stop buffer (2) was added, and samples were

* Corresponding author.

extracted twice with phenol-chloroform prior to ethanol coprecipitation with glycogen and analysis on a standard 6% polyacrylamide-urea sequencing gel.

UV cross-linking. Bromodeoxyuridine-substituted probes were prepared essentially as described previously (2), except that the single-stranded template was derived by cloning the binding sites into pBluescript and rescuing by superinfection with VCSM13 under the manufacturer's conditions (Stratagene). After performing standard binding reactions, samples were UV irradiated for ~10 to 14 min under a National Biologic Corp. (Twinsburg, Ohio) model 1100A illuminator as a drop on Parafilm placed on ice. An equal volume of 2× sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis loading buffer was added, and the sample was heated at 70°C for 5 min and loaded on a standard SDS-polyacrylamide gel. Alternatively, gel mobility shifted complexes were excised and the gel slice was irradiated for 20 min, followed by elution into 0.3 M NaAc-1 mM EDTA-0.2% SDS.

RESULTS

Identification of the major enhancer element of the type IV collagenase gene. Previously, we found that 3.5 kbp of 5' flanking sequence from the T4 gene possessed strong transcriptional activity when transiently transfected into HT1080 cells (8). Deletion analysis (Fig. 1a) was performed on an equally active 2.4-kbp fragment to localize major sites of enhancer activity. Removal of the region spanning -1910 to -1635 abolished about two-thirds of the transcription from CAT constructs transiently transfected into HT1080 fibrosarcoma cells (20). When this 275-bp region was cloned into a CAT vector upstream of a thymidine kinase promoter, it was found to enhance transcription (Table 1).

To localize sites of DNA-protein interaction in this fragment, DNase I protection assays were performed with nuclear extracts (6) from HT1080 cells (Fig. 1b). Two major protected regions, r1 and r2, were found. Double-stranded oligonucleotides corresponding to r1 and r2 were then assayed for enhancer activity in three promoter contexts (Table 1): a 400-bp promoter-proximal fragment from the T4 gene, the thymidine kinase promoter, and a 65-bp minimal promoter derived from the interstitial collagenase gene (and lacking the AP-1 binding site). The r2 oligonucleotide enhanced significantly in all three contexts, indicating that it could enhance transcription from both TATA-containing (tk, collagenase) and TATA-less (T4) promoters. The 400-bp T4 promoter-proximal fragment without r2 gave about the same transcription level as did the enhancerless thymidine kinase promoter. Addition of r2 to the former fragment gave about the same transcription as the complete 2.4-kbp T4 genomic fragment, suggesting that sequences in the 400-bp fragment contained only core promoter elements, the region -1635 to -400 was dispensable, and the r2 enhancer was essential for efficient transcription.

r2 binds a protein whose specificity is similar to that of AP-2. The r2 enhancer sequence has 11/15 homology (Fig. 2a) with the AP-2 binding site of simian virus 40 (SV40) early enhancer (26), allowing one 3-bp gap. To test the possibility that the protein interacting with r2 resembled AP-2 in its binding specificity, several experiments were performed.

First, double-stranded oligonucleotides bearing AP-2 or other documented binding sites were tested for ability to compete against formation of r2 DNA-protein complexes, as assayed by gel mobility shift experiments. Of the five sequences tested, only one, corresponding to an AP-2 binding

site, competed against complex formation (Fig. 2b). The AP-2 binding site used as a competitor in the experiment shown was derived from the metallothionein hMTIIa gene (9). The sequence of this binding site differs substantially from that of the AP-2 site of SV40 (26) (with which the r2 binding site is homologous), yet it still competes effectively against binding of the r2 factor, strengthening the similarity between these two factors.

Second, purified recombinant AP-2 protein footprinted r2 when the r2-containing fragment representing -1635 to -1950 was used as a probe (Fig. 2c). The region protected by AP-2 binding was less extensive in the 5' noncoding direction than the region protected by HT1080 nuclear extract proteins, but whether this is due to additional proteins interacting at this site in the latter case or to artifactual differences resulting from bacterial expression of the former is unclear. Also, a much larger number of DNase I-hypersensitive sites is generated by AP-2 than by nuclear extract. Nevertheless, the AP-2 protein was capable of selectively binding to the r2 site.

r2 enhancer is E1A repressible. We reported previously that the endogenous T4 gene was repressed in HT1080 cells by stable transfection of the E1A gene and that a 3.5-kbp promoter fragment was repressed by cotransfection with E1A expression plasmids (8).

To determine whether the r2 enhancer is sensitive to E1A, transient cotransfection experiments were performed with this enhancer, in combination with either the thymidine kinase or the T4 (400-bp) promoter fragment (Table 2). Plasmids expressing either the 13S or the 12S form of E1A repressed transcription in the r2-tk promoter combination by 2.8- and 5.6-fold, respectively, and very similar repression (3.9- and 5.5-fold) was obtained with the r2-minimal T4 promoter reporter plasmid. Neither of the minimal, enhancerless promoters was sensitive to E1A repression (8). The magnitude of the repression was similar to that of the endogenous T4 gene, suggesting that the r2 enhancer is a major target for repression by E1A in this gene. A small internal deletion in E1A domain 1 that abolishes binding of the protein to retinoblastoma protein (25; E. Ziff, personal communication) still repressed the enhancer as efficiently as the parental 13S cDNA. This result suggests that retinoblastoma protein may not be involved in the repression of the r2 enhancer by E1A, in agreement with other mutational analysis performed with respect to AP-1 inhibition (8, 24).

Evidence for a two-element silencer located adjacent to the r2 enhancer. When transfected transiently into mcf-7 mammary carcinoma cells, the r2 enhancer sequence alone was found to be about 20-fold more active than the 300-bp genomic fragment from which it was derived. This suggested the presence of negative elements or silencers, as have been described for several cellular genes (3, 7, 10, 23).

To locate the position of a putative silencer, 5' deletions were made upstream of the r2 sequence, assuming that the footprint r1 could be due to the silencer. Surprisingly, inclusion of even the complete region 250 bp upstream of r2 had minimal or no effect on transcription (Fig. 3). In contrast, inclusion of the 40-bp sequence located immediately downstream of r2 in the T4 gene repressed transcription by about 11-fold. This implicated the 40-bp sequence as a silencer. The rule out the possibility that transcription was being inhibited by simply distancing the enhancer from the start site, a polylinker sequence of similar length was substituted, with only minimal effect on transcription.

To characterize the silencer element(s) more precisely, several subfragments of the 40-bp silencer were tested for

TABLE 1. Enhancer activity of r2 in three core promoter contexts^a

Plasmid	Enhancer	% Conversion	Fold enhancement
T4 promoter			
pT4Catpro		4.7	(1)
pT4Cat5	1,910-bp fragment	47.3	10
pT4Catr2sipro	r2 + 45 bp downstream	46.8	10
tk promoter			
pBLCat2		4.3	(1)
pT4Catf1	-1910-1635	53.6	12.5
pT4CatΔHB	-1735-1635	61.7	14.3
pT4Catr2m	r2	76.0	>17.7
CL promoter			
pCLΔAp1		0.1	(1)
pr2CL1-1	r2	12.4	124

^a Expressed as fold enhancement over the core promoter basal transcription.

silencer activity in the context of the r2 enhancer with tk promoter. First, inspection of the DNA sequence revealed the presence of a tandemly duplicated 10-bp sequence, which was imperfectly repeated at only 2 bp (AG versus GA) (Fig. 3). When this tandem repeat (x1 + x2) was tested for silencer activity, it was found to repress by 4-fold; insertion of an additional copy of x1 increased its inhibitory effect to 21-fold. This identifies the 10-bp "x-box" as a minimal silencer element whose activity is revealed when multimerized.

The remaining 20 bp of the region immediately downstream of x1 - x2 (siΔ3) was then tested for silencer activity (Fig. 3) and was found to be capable of silencing, independently of x1 or x2, by about 10.7-fold. These combined results show the presence of two silencer elements within this 40-bp region. In a construct configured r2-x1-x1-x2-siΔ3-tk promoter, 56-fold inhibition was obtained relative to r2-tk. This configuration only differs from that in the T4 gene by one additional x1 box, which has the effect of increasing repression by five- to sixfold. Placement of the silencer upstream of r2-tk weakened its activity slightly (Fig. 3, last line).

When assayed in HT1080 cells in the context of r2-tk, the silencer effect was four- to sixfold weaker than in mcf-7 cells, depending on which construct was used. The effects of both x1/x2 boxes and of siΔ3 sequences were reduced (Fig. 3). This result suggests that the silencer may act in a cell type-specific fashion to quench transcription of the T4 gene.

Interaction of the T4 silencer with heterologous enhancer or core promoter. The T4 silencer could potentially repress by interfering with enhancer elements, core promoter elements, or communication between the two. To determine which mechanism applied, the enhancer element was first changed from r2 to an AP-1 site. Silencer activity in this context (Fig. 4) was very potent (~140-fold for the AP-1-x1-x1-x2-siΔ3-tk construct in mcf-7 cells). As with the r2 enhancer, the silencer was far less effective when assayed in HT1080 cells (~3-fold inhibition).

Silencer activity was also tested in the context of the T4 promoter itself to determine its relevance to the function of this gene. Because T4 core promoter sequences have not been precisely defined, a 400-bp promoter-proximal fragment capable of promoting basal transcription at a level comparable to that of the tk promoter was used. In the configuration r2-x1-x1-x2-siΔ3-T4 promoter, the silencer re-

pressed by about sixfold. While this is about ninefold less potent inhibition than that obtained in the tk promoter background, it is unclear whether this weakness is due to the 400-bp distance to the start site or to a weaker interaction of the silencer with T4 core promoter elements. Nevertheless, the results indicate that the silencer can function at a distance and in the context of a TATA-less promoter such as the T4 promoter.

The hypothesis that the silencer interferes with core promoter function was tested by assaying its effect on tk promoter basal (enhancerless) transcription. In this context, the silencer (configuration x1-x2-siΔ3) repressed by about sixfold, confirming that either core promoter or promoter-proximal Sp1 and CTF factors were inactivated by the silencer.

Identification of proteins binding to the T4 silencer. The strategy used to identify proteins binding specifically to silencer elements was to point mutate the individual elements, assay the mutant elements for silencer function, and then compare the abilities of wild-type (WT) and mutant DNA fragments to form gel mobility shift complexes.

The mutational analysis of the silencer elements (Table 3) was performed by introducing 3-bp mutations (mut) into the corresponding positions of x1 and x2 and assaying them in the context r2-x1(WT)-x1(mut)-x2(mut)-tk or introducing 5-bp mutations into siΔ3 and assaying them in the context r2-siΔ3-tk. For the x-boxes, the upstream-most mutation (m1) had the least (~3-fold) effect, and the middle and downstream mutations m2 and m3 had a greater (6- to 10-fold) effect on silencer function. For the siΔ3 element, the wild-type sequence itself repressed by 7-fold, whereas mutants 2, 3, and 4 repressed by only 1.7-, 2.1-, and 2.7-fold, respectively.

The effects of these mutations were then tested in the context of the complete silencer, in the configuration r2-x1(mut2)-x2(mut2)-siΔ3-tk or r2-x1(WT)-x2(WT)-siΔ3(mut2) (Table 3). In this context, neither mutation affected silencer function. These results (and the data in Fig. 3) suggest that the two elements do not silence cooperatively; instead, they appear to reflect redundant binding sites for the same protein or mutually exclusive binding sites for two different proteins.

Proteins from mcf-7 whole-cell extracts (16) were fractionated on heparin-Sepharose. Proteins eluting at 0.1 and 0.45 M KCl gave detectable complexes on gel mobility shift gels, using end-labeled silencer probes. Complexes formed on siΔ3 element oligonucleotides with 0.1 M (heparin flow-through) protein were specific by two criteria: (i) formation on wild-type siΔ3 but not siΔ3mut2 probes (Fig. 5a); (ii) competition by unlabeled wild-type but not siΔ3mut2 competitor DNA at 50-fold excess (Fig. 5b).

A more slowly migrating, but more intense complex was formed by using the heparin 0.45 M protein and a complete silencer (x1-x2-siΔ3) probe (Fig. 5c). This complex could not be inhibited by a 150-fold excess of either wild-type or mutant siΔ3, suggesting that its binding site includes sequences upstream of siΔ3. Complete silencer oligonucleotides could compete against formation of this complex, as could the complete silencer bearing the siΔ3-inactivating mutation 2, but the complete silencer bearing mutation 2 in both x-boxes failed to compete. The protein responsible for this complex presumably bound x1 or x2 or both; whether siΔ3 sequences are also bound is unclear.

To initiate an identification of this protein, complexes were formed by using a complete silencer probe which had been synthesized by copying a single-stranded template with DNA polymerase in the presence of bromodeoxyuridine-

FIG. 2. r2 enhancer is a potential AP-2 binding site. (a) Sequence homology between r2 and the AP-2 site of SV40. (b) AP-2 binding sites compete against r2 complex formation, using HT1080 nuclear extract. Nuclear protein was preincubated with 100-fold excess of indicated double-stranded oligonucleotide prior to addition of end-labeled r2 probe and gel mobility shift analysis. Competitor sequences are indicated in Materials and Methods. (c) Purified, recombinant AP-2 footprints r2. 0.2 footprint unit of AP-2 protein (R. Tjian laboratory) was incubated under the same binding conditions as in Materials and Methods, using an end-labeled probe including sequences -1635 to -1950, except pIdC was omitted.

triphosphate in place of dTTP and labeled triphosphates. After binding, the reaction was UV irradiated to cross-link the DNA to protein and analyzed on an SDS-polyacrylamide gel. A single protein of ~185 kDa was labeled by this procedure (Fig. 5d). It exhibited the same competition profile as the gel mobility shift complex (Fig. 5c), suggesting that the complex involves binding of one or more molecules of this single protein species.

DISCUSSION

Type IV collagenase has been implicated in the basement membrane degradation accompanying tumor invasion and

metastasis (8, 12, 13, 18). Tissue remodeling such as the implantation of mouse blastocysts in the uterine wall (4) or at many other stages of embryogenesis may frequently require secreted proteases. This raises the question of how the protease genes are regulated. While the interstitial collagenase gene is induced rapidly by various growth factors (EGF, PDGF, interleukin-1), by phorbol esters, and by various oncogenes (*src*, *ras*, *mos*) (see reference 17 for a review), the T4 gene is not apparently growth factor inducible (5; unpublished observations). The T4 gene is induced by *ras* transformation in primary human bronchial epithelial cells (5), but attempts to recapitulate this effect in transient

TABLE 2. Evidence that r2 enhancer is E1A repressible^a

Reporter plasmid	E1A plasmid	% Conversion	Fold Inhibition
pT4Catr2Bam	pE1Afs	18.9	(1)
	pSVXL3	22.7	1
	pSVF12	3.4	5.6
	pSVN20	6.7	2.8
	pSVXL105	6.6	2.9
pT4Catr2pro	pE1Afs	7.1	(1)
	pSVXL3	9.1	0.8
	pSVF12	1.3	5.5
	pSVN20	1.8	3.9
	pSVXL105	2.4	3.0

^a Cotransfection experiments were performed as described previously (8). The reporter plasmids contained the r2 enhancer linked to a thymidine kinase promoter (pT4Catr2Bam) or to a 400-bp promoter-proximal T4 gene fragment (pT4Catpro). E1A plasmids were described before (22). pE1Afs, Frame-shifted E1A control coding for first three amino acids; pSVXL3, frame-shifted control coding for first 70 amino acids; pSVF12, 12S E1A cDNA; pSVN20, 13S E1A cDNA; pSVXL105, 13S cDNA from which nucleotides 671 to 692 have been deleted.

transfection experiments have been unsuccessful. However, the T4 gene is repressible by the E1A oncogene of adenovirus (8). In addition, it does seem to show some degree of tissue specificity in that fibroblasts or fibrosarcoma cells express the gene (5, 8), in contrast to epithelial cell lines, such as mcf-7 or HeLa (unpublished data). Experiments

FIG. 3. Identification by transient transfection analysis of a silencer composed of two elements. All constructs contained T4 sequences upstream of a thymidine kinase promoter in the vector pBLCat2. Sequence symbols are defined at the bottom. A 1.2-μg amount of test plasmid was transfected per 60-mm dish, with 2.9 μg of pBluescript carrier, into mcf-7 or HT1080 cells. *Expression below vector background level.

FIG. 4. T4 silencer functions in the context of AP-1 enhancer-tk promoter, or in the context of r2-T4 promoter, and inhibits transcription from the enhancerless tk promoter. Transfections were performed as in the footnote to Table 3. The AP-1 binding site used was AGCTTGACTCAGCGAT. Sequence symbols are defined at the bottom. *Expression below vector background level.

presented herein analyze the DNA elements contributing to basal expression, E1A repressibility, and cell type specificity.

E1A repressibility of the T4 gene. The major enhancer, r2, of the T4 gene was found to be similar to an AP-2 element by two criteria of binding specificity: gel mobility shift competition and footprinting with purified AP-2 protein. Protein isolation will be necessary to demonstrate whether the

TABLE 3. Effect of point mutations on silencer function^a

Plasmid	Sequence	Fold inhibition
siΔ1	ACTGAAGCCCACTGAGACCCAG	23
siΔ1m1	AGT AGT	6.3
siΔ1m2	CCT CCT	2.5
siΔ1m3	AGG AGG	3.7
siΔ3	AGCCGCAGAGACTTTTCTAGCTGTGAG	7
siΔ3m1	TAATA	6.1
siΔ3m2	CTCAG	1.7
siΔ3m3	GAGCT	2.1
siΔ3m4	TGTC	2.7
six	(siΔ1siΔ3)	27
sixm1	(siΔ1m1siΔ3)	25
sixm2	(siΔ1siΔ3m2)	25

^a Individual silencer elements were assayed for silencer function in the context r2-x1(WT)-x1(mut)-x2(mut)-tk or r2-siΔ3(mut)-tk. Fold inhibition is relative to r2-tk. The three bottom lines represent plasmids bearing complete silencers, with mutations in either the x-boxes or the siΔ3 boxes.

FIG. 5. Identification of proteins interacting with the silencer elements. (a) Gel mobility shift analysis of mcf-7 heparin flowthrough protein bound to siΔ3(WT) or siΔ3(mut2) probes. (b) Competition analysis of siΔ3 complex, inhibited with 100-fold excess of unlabeled double-stranded oligonucleotides as indicated. siΔ1 = x-box competitor; six = complete silencer competitor; others as defined previously. (c) Competition analysis of a complex formed with complete silencer probe (bromodeoxyuridine-substituted probe generated by Klenow polymerase reaction on single-stranded template) with heparin-0.45 M eluate. Competitor names are as defined in Table 3. (d) Identification of the protein involved in the complexes shown in panel c by UV cross-linking. Aliquots of the binding reactions performed in panel c were UV irradiated and analyzed on SDS-polyacrylamide gel electrophoresis.

protein interacting with r2 is identical to AP-2; however, the cloned 54-kDa AP-2 gene appears to encode a unique factor rather than a member of a family of homologous transcription factors (26).

In cotransfection experiments, the repression of the r2 enhancer by E1A was of similar magnitude to the repression of the whole T4 regulatory region, suggesting that the r2 enhancer is a major target for repression by E1A; however, it may not be the only target. The mechanism of repression of r2 (or of any other enhancer element) by E1A remains to be solved. AP-2 protein forms a nonbinding complex with SV40 T antigen that may be important in autoregulation of

the SV40 early enhancer (26). While T antigen and E1A both bind retinoblastoma protein, mutation of the Rb protein-binding domain of E1A did not disable its repression function (Table 2), suggesting that Rb protein is not directly involved in repression. This is in agreement with observations on the E1A repression of the stromelysin gene (24). Furthermore, the level of r2-binding activity in E1A-expressing HT1080 cells was normal, suggesting that repression occurs at the level of transcriptional activation, not binding.

Identification of the T4 silencer. The contribution of negative regulation to both transient and tissue-specific gene regulation is being increasingly recognized (11, 21). The

FIG. 5—Continued

identification of the T4 silencer provides a new model system in which to examine the mechanism of transcriptional repression.

Transcriptional silencers have been found in a number of cellular genes, including *c-myc* (23), *c-fos* (14), vimentin (7), lysozyme (3), and the T-cell receptor alpha chain gene (27). It is possible to envision that a bound silencer protein could repress transcription by interfering at a distance with core promoter factors, by interfering with the enhancer itself, or by interfering with enhancer-core promoter communication. The behavior of the T4 silencer is most consistent with the first model, since core promoter function can be repressed in the absence of an enhancer; similar behavior has been shown for the vimentin silencer. Purification of the silencer-binding protein(s) will allow molecular analysis of its specific effects on core promoter complex formation and function.

The T4 silencer exhibits a modular array of functional

elements (two x-box elements, one siΔ3 element), one of which cannot silence as a monomer. This behavior is similar to that of enhancers, as described in the SV40 early promoter (19), except with an opposite effect on transcription. Similarly, the lysozyme silencer also has a modular structure (3). Conceivably, repression of transcription could be achieved differentially in different cell types having one, both, or neither of the proteins interacting with these elements. Neither of the T4 silencer elements functions efficiently in HT1080 fibrosarcoma cells, a T4-producing cell line, while both elements function well in mcf-7 cells, a T4-nonproducing cell line. This suggests that cell type-specific expression of the T4 gene may be partly determined by the silencer. Preliminary experiments (Frisch, unpublished data) have indicated that the 185-kDa protein may be present in HT1080 cells, implying that a mechanism more subtle than simple regulation of silencer protein concentra-

tion may apply; future studies will be directed toward elucidating this mechanism.

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