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Control of Cytochrome P_1-450 Gene Expression by Dioxin

Abstract. *The environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) may produce its effects by altering gene expression in susceptible cells. In mouse hepatoma cells, TCDD induces the transcription of the cytochrome P_1-450 gene, whose product, aryl hydrocarbon hydroxylase, contributes both to the detoxification and to the metabolic activation of carcinogenic polycyclic aromatic hydrocarbons. A DNA fragment containing sequences flanking the 5′ end of the cytochrome P_1-450 gene was isolated and analyzed. This DNA fragment contains a* cis*-acting control element with at least three functional domains: a putative promoter, an inhibitory domain upstream from the promoter that blocks its function, and a TCDD-responsive domain still farther (1265 to 1535 base pairs) upstream of the promoter. These findings, together with results from earlier studies, imply that transcription of the cytochrome P_1-450 gene is under both positive and negative control by at least two* trans*-acting regulatory factors.*

Halogenated dibenzodioxins have generated interest because of their potential toxicity and their presence as environmental contaminants. Effects of the prototypical dioxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), such as epithelial metaplasia, tumor promotion, teratogenesis, and enzyme induction, suggest that the compound alters gene expression in responsive cells (*1*). The binding of TCDD to an intracellular protein receptor and the accumulation of TCDD-receptor complexes in the nucleus are required for TCDD action (*1, 2*). We have studied the mechanism by which TCDD increases the expression of the cytochrome P_1-450 gene in mouse hepatoma cells. In wild-type (Hepa 1c1c7) cells, TCDD induces about a 20-fold increase in the rate of transcription of the cytochrome P_1-450 gene, which is followed by corresponding increases in the content of cytochrome P_1-450 messenger RNA (mRNA) and in the activity of aryl hydrocarbon hydroxylase (E.C. 1.14.14.1) (*3, 4*).

We have isolated two classes of variant cells that show decreased responsiveness to TCDD (*3, 5*). Class I variants form few TCDD-receptor complexes; those that do form accumulate within the nucleus, as is the case with wild-type cells. These variant cells show decreased accumulation of cytochrome P_1-450 mRNA and lower aryl hydrocarbon hydroxylase activity in response to TCDD. Class II variants form a normal number of TCDD-receptor complexes; however, the complexes do not accumulate in the nucleus. These variant cells do not transcribe the cytochrome P_1-450 gene in response to TCDD. Thus, induction of cytochrome P_1-450 gene transcription by TCDD requires the localization of TCDD-receptor complexes in the nucleus.

The results of nuclear transcription assays indicate that concurrent exposure to TCDD and cycloheximide superinduces the rate of transcription of the cytochrome P_1-450 gene to a level about ten times greater than the maximum level induced by TCDD alone. Superinduction requires functional TCDD-receptor complexes because it does not occur in class II variant cells (*6*). These findings indicate the existence of a second control mechanism for cytochrome P_1-450 gene transcription. Taken together, our results imply that at least two *trans*-acting factors regulate cytochrome P_1-450 gene expression: (i) the TCDD-receptor complex and (ii) a labile repressor protein that negatively modulates the action of the TCDD-receptor complex.

We have isolated a third class of variant cells that overtranscribe the cytochrome P_1-450 gene in response to TCDD. These cells are designated high activity variant (HAV) cells. Our analyses suggest that these variants contain an altered *cis*-acting genomic element that increases their responsiveness to TCDD (*7*). We have isolated from HAV cells a genomic element that regulates the response of the cytochrome P_1-450 gene to TCDD. This control element has novel properties compared to other elements known to regulate the expression of eukaryotic genes.

A genomic library was constructed by partial digestion of HAV cellular DNA with Mbo I, insertion of 10- to 16-kb (kilobase) fragments into the Bam HI site in bacteriophage λ L47.1, and packaging in vitro (*8*). After growth on *Escherichia coli* C600, plaques containing cytochrome P_1-450 DNA were isolated by repetitive screening with nick-translated cytochrome P_1-450 complementary DNA (*9*). Figure 1A shows the restriction map of one clone, designated λ CPM 17. Hybridization of nick-translated cytochrome P_1-450 cDNA to restriction digests of λ CPM 17 and hybridization of nick-translated λ CPM 17 restriction fragments to RNA from uninduced and TCDD-induced cells determined the orientation of the fragments within the clone and identified a 2.58-kb Hind III fragment that appeared to contain the 5′ end of cytochrome P_1-450 gene.

To determine whether the 2.58-kb Hind III fragment contains regulatory information, we inserted it into the plasmid pSV0cat at the Hind III site immediately upstream of the chloramphenicol acetyltransferase (CAT) gene (*10*). Recombinants were isolated as ampicillin-resistant colonies in *E. coli* HB101, and the

orientation of the insert was determined by restriction analysis. The plasmid, designated pHAVcat, is the recombinant containing the insert in the correct 5′ to 3′ orientation (that is, the 3′ end of the 2.58-kb fragment is immediately upstream of the CAT gene). We transfected pHAVcat into wild-type cells using a combination of techniques (*11*). As a positive control most experiments included cells transfected with pSV2cat, in which the simian virus 40 enhancer-promoter element drives expression of the CAT gene (*10*). CAT activity was measured by a modification of the procedure described (*10*).

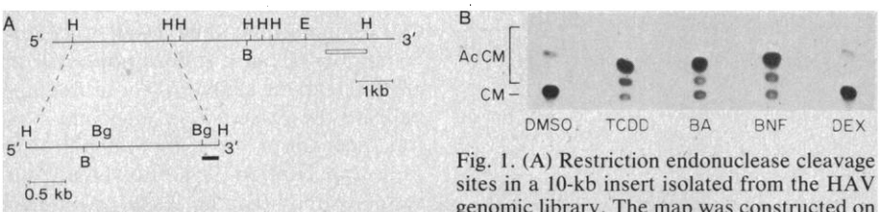


Fig. 1. (A) Restriction endonuclease cleavage sites in a 10-kb insert isolated from the HAV genomic library. The map was constructed on the basis of digestions with Eco RI (E), Hind III (H), Bam HI (B), and Bgl II (Bg) either separately or in combination. The open rectangle indicates the location of the sequence that hybridizes to cytochrome P_1-450 complementary DNA. The black rectangle indicates the 220-bp Hind III–Bgl II fragment used in the primer extension experiments. The 2.58-kb Hind III fragment was used to construct the plasmid pHAVcat. (B) Expression of CAT activity in pHAVcat-transfected cells exposed to maximum-inducing concentrations of TCDD, benz[*a*]anthracene (BA), β-naphthoflavone (BNF), or dexamethasone (DEX), each dissolved in dimethyl sulfoxide (DMSO) (*26*). CAT activity was determined by measuring the conversion of [^{14}C]chloramphenicol (CM) to acetylated metabolites (AcCM) under conditions linear with respect to both time and amount of cell extract (*27*). Each experiment was performed at least three times with consistent results.

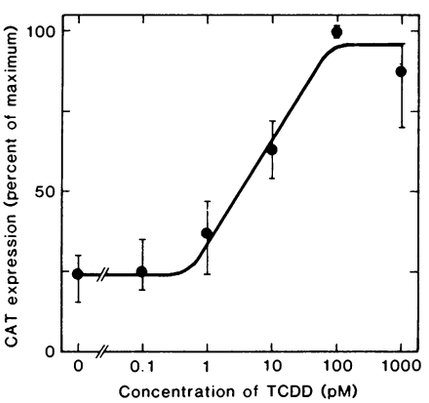


Fig. 2. Induction of CAT activity in pHAVcat-transfected cells by TCDD. Wild-type cells were transfected with pHAVcat as described (*26*) and were exposed to the indicated concentrations of TCDD. After visualizing the results of the CAT assays by autoradiography, areas containing chloramphenicol and its acetylated metabolites were cut from the silica plates and measured by scintillation counting. Points indicate average values from three experiments; brackets indicate the range of values.

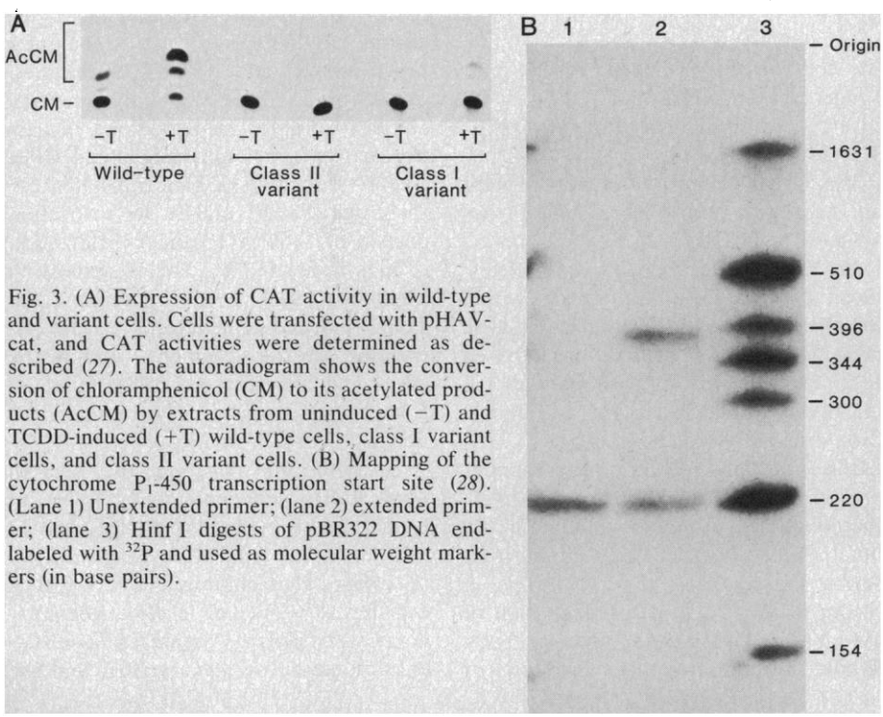


Fig. 3. (A) Expression of CAT activity in wild-type and variant cells. Cells were transfected with pHAVcat, and CAT activities were determined as described (*27*). The autoradiogram shows the conversion of chloramphenicol (CM) to its acetylated products (AcCM) by extracts from uninduced (−T) and TCDD-induced (+T) wild-type cells, class I variant cells, and class II variant cells. (B) Mapping of the cytochrome P_1-450 transcription start site (*28*). (Lane 1) Unextended primer; (lane 2) extended primer; (lane 3) Hinf I digests of pBR322 DNA end-labeled with ^{32}P and used as molecular weight markers (in base pairs).

TCDD induced CAT expression in wild-type cells transfected with pHAVcat (Fig. 1B). Thus, a DNA element flanking the 5′ end of the cytochrome P_1-450 gene can place a heterologous gene under TCDD control. The presence of CAT activity in uninduced cells transfected with pHAVcat is consistent with the presence of cytochrome P_1-450 mRNA in uninduced HAV cells (*7*). Both phenomena are probably the result of endogenous inducers in the culture medium (*7*). Wild-type cells transfected with pSV0cat containing no insert did not express CAT activity. Other compounds, such as benz[*a*]anthracene and β-naphthoflavone, that induce cytochrome P_1-450 gene expression (*2*) also induced CAT expression, whereas a compound (dexamethasone) that does not induce cytochrome P_1-450 gene expression (*2*) did not induce CAT expression. Thus, the induction of CAT expression had the correct inducer specificity.

In pHAVcat-transfected wild-type cells, TCDD induced CAT expression in a dose-dependent manner, with a median effective dose (ED_{50}) of about 5 p*M* (Fig. 2). This is similar to the ED_{50} for the induction of cytochrome P_1-450 gene expression in HAV cells (*7*). Thus, the induction of CAT expression had the expected sensitivity to TCDD.

Transfection of pHAVcat into class I and class II variant cells showed that TCDD induced little CAT expression in the class I variants and no detectable CAT expression in the class II variants (Fig. 3A). These responses of the CAT gene to TCDD parallel those of the cytochrome P_1-450 gene in the variants (*3*, *4*). Thus, induction of CAT expression in the transfected cells required functional TCDD-receptor complexes. Control experiments with pSV2cat indicated that the variant cells were not more resistant to transfection than wild-type cells.

The results of our transfection experiments indicate that the induction of CAT expression has the expected inducer specificity, has the expected sensitivity to TCDD, and requires functional TCDD-receptor complexes. These observations imply that the 2.58-kb Hind III fragment contains an element that responds to TCDD-receptor complexes and controls the expression of the gene downstream.

We used primer extension analysis (*12*) to locate the start site of transcription. The primer, the 220-bp (base pair) Hind III–Bgl II fragment at the 3′ end of the 2.58-kb Hind III fragment (Fig. 1A), was end-labeled with ^{32}P, hybridized to

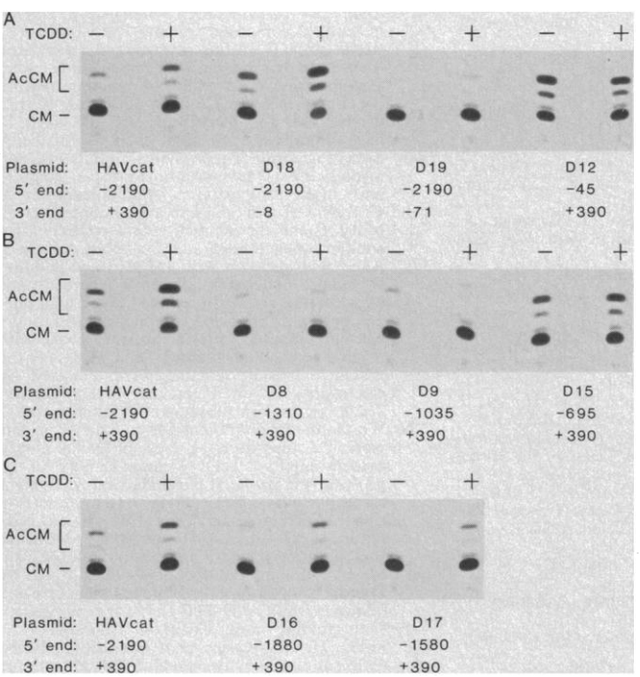
polyadenylated RNA from TCDD-induced HAV cells, and extended with reverse transcriptase. The resulting DNA fragment was analyzed by denaturing gel electrophoresis and autoradiography. The extended primer had a length of 390 ± 5 bp (Fig. 3B). Thus, the start site of transcription is about 390 bp from the 3′ end of the 2.58-kb Hind III fragment. For simplicity, the transcription start site is designated as position 0 along the 2.58-kb Hind III fragment. Nucleotides upstream (5′) of this position are denoted by consecutive negative (−) numbers and those downstream by consecutive positive (+) numbers.

To prepare plasmids for deletion analysis, we subcloned the 2.58-kb Hind III fragment into plasmid pBR322 and isolated recombinants that contained the insert in either orientation. The recombinants were linearized and then digested for various times with Bal 31 exonuclease (*13*). The ends were repaired and Hind III linkers were added; the recombinants were then digested with Hind III, and the shortened HAV DNA inserts were recovered after electrophoresis in agarose gels (*14*). The shortened inserts were subcloned into pSV0cat, and their size and orientation were determined by restriction analysis. Recombinants were transfected into wild-type cells, and CAT expression was analyzed in the absence and presence of TCDD. Each experiment included cells that had been transfected with pHAVcat for comparison. Figure 4 shows representative autoradiograms from these experiments, and the constructs are summarized in Fig. 5A. The sizes of the deletion fragments were determined by restriction analyses; each value has an error of about 1 percent.

To locate a putative promoter domain, we analyzed deletions near the start site of transcription. Cells transfected with D18, containing nucleotides −2190 to −8, expressed TCDD-inducible CAT activity (Fig. 4A). The levels of CAT activity in both uninduced and TCDD-induced cells were greater than those in pHAVcat-transfected cells. Because the translational start site for cytochrome P_1-450 in mouse liver is about 100 bp 3′ of the transcriptional start site (*15*), pHAVcat probably contains translational start sites for both the cytochrome P_1-450 gene and the CAT gene. Thus, cells transfected with D18, which contains only the CAT gene translational start site, may express CAT more efficiently than cells transfected with pHAVcat. In contrast, cells transfected with D19, which contains nucleotides −2190 to −71, did not express CAT activity. Thus, a DNA sequence between nucleotides −71 and −8 is required for CAT expression. In addition, cells transfected with D12, which contains nucleotides −45 to +390, constitutively expressed CAT activity. Thus, a DNA sequence (or sequences) necessary for transcription is between nucleotides −45 and −8. This putative cytochrome P_1-450 promoter has a relatively high efficiency, as indicated by the high constitutive expression of CAT in cells transfected with D12.

Cells transfected with D15 (nucleotides −695 to +390) also constitutively expressed CAT activity (Fig. 4B). A 650-bp segment of DNA (nucleotides −695 to −45) upstream of the promoter had no detectable effect on CAT expression, and its function remains unknown. In contrast, cells transfected with D9 (nucleotides −1035 to +390) or D8 (nucleotides −1310 to +390) expressed little constitutive and no TCDD-inducible CAT activity (Fig. 4B). These findings imply the existence of a DNA domain upstream of the promoter between nucleotides −1310 to −695 that inhibits promoter function.

Cells transfected with D17 (nucleotides −1580 to +390) expressed CAT activity in response to TCDD (Fig. 4C). This observation implies the existence of a DNA domain between nucleotides −1580 and −1310 that responds to TCDD, presumably by interacting with the TCDD-receptor complex. Compared to cells transfected with D17, cells transfected with D16 (nucleotides −1880 to +390) showed an increase in CAT expression. Cells transfected with pHAVcat (nucleotides −2190 to +390) showed a further increase in CAT expression (Fig. 4C). These results indicate that DNA sequences between nucleotides −1880 and −1580 and between



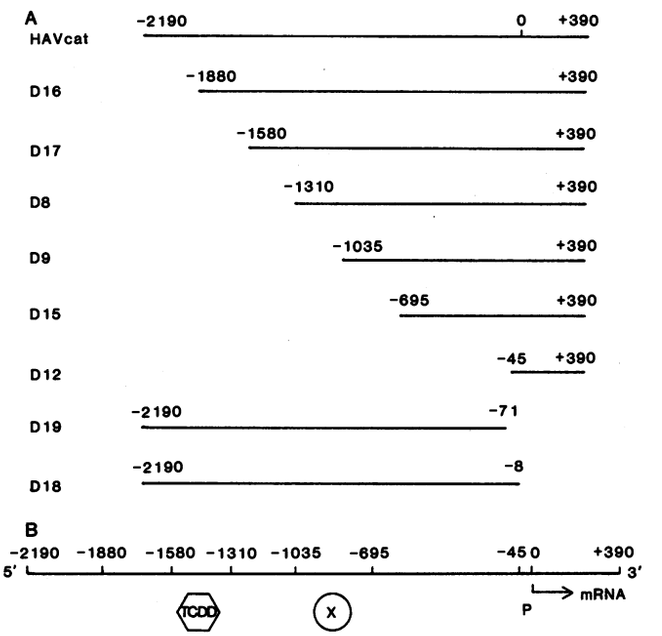


Fig. 4 (left). Expression of CAT activity in wild-type cells transfected with deletion plasmids. Deletion plasmids were constructed as described in the text. Wild-type cells were transfected with the indicated plasmids, and CAT assays were performed on extracts from uninduced (−TCDD) and induced (+TCDD) cells. The limits of the DNA insert in each plasmid are noted below the autoradiograms. Fig. 5 (right). (A) Summary of deletion constructs. (B) Genomic regulatory element for the cytochrome P_1-450 gene. Hexagon containing TCDD represents a TCDD-receptor complex. The circle containing X represents a hypothetical repressor protein; P indicates the presumed promoter region.

nucleotides −2190 and −1880 increase the efficiency of TCDD-induced CAT expression.

Our results indicate that the *cis*-acting genomic control element flanking the 5′ end of the cytochrome P_1-450 gene in HAV cells contains at least three functional domains: (i) a putative promoter near the transcription start site; (ii) an inhibitory domain 650 to 990 bp upstream from the promoter; and (iii) a TCDD-responsive domain 1265 to 1535 bp upstream from the promoter (Fig. 5B). Both the location of the domain immediately upstream of the transcription start site and its activity in the absence of TCDD suggest that it contains the promoter for the cytochrome P_1-450 gene. Its relatively high efficiency represents a functional difference between the putative promoter for cytochrome P_1-450 in HAV cells and promoters for other inducible eukaryotic genes (*16*).

The mechanism by which the inhibitory domain reduces promoter efficiency might include the binding of a negative regulatory protein to a site or sites within the inhibitory domain. Decreased binding of a labile repressor protein to this domain might account for the superinduction of gene transcription by TCDD and cycloheximide (*6*). Inhibitory elements may also influence expression of the human β-interferon gene (*17*), of the c-*myc* gene in Burkitt's lymphoma (*18*), and of the yeast cytochrome c gene (*19*).

Compared to other examples of *cis*-acting regulatory regions, the location of the TCDD-responsive element 1265 to 1535 bp upstream of the start site of transcription is novel (*20*). Because the TCDD-responsive element controls gene expression from such a distance, it may be a TCDD-regulated enhancer element (*21*). Our deletion analyses suggest that there might be at least three TCDD-responsive elements, each of which might be a binding site for the TCDD-receptor complex. There is evidence for multiple binding sites for steroid-receptor complexes upstream of steroid-inducible genes (*22–24*). An alternative possibility is that there is one binding site for the TCDD-receptor complex and that DNA sequences upstream from this site augment the signal that activates gene transcription.

Results from earlier studies imply that at least two regulatory factors control expression of the cytochrome P_1-450 gene (*3–6*). The TCDD-receptor complex is the primary factor; it increases the rate of transcription of the cytochrome P_1-450 gene. The findings from our superinduction experiments suggest the existence of a labile repressor protein that inhibits transcription of the cytochrome P_1-450 gene. The present results indicate that at least two DNA elements control cytochrome P_1-450 promoter function. One is a TCDD-responsive element, which presumably interacts with the TCDD-receptor complex; the other is an inhibitory element, which might interact with a cycloheximide-sensitive repressor. Our working hypothesis is that the binding of the TCDD-receptor complex to its regulatory domain releases the efficient cytochrome P_1-450 promoter from inhibition. This basic mechanism differs from those proposed for other eukaryotic systems in which a receptor mediates the induction of gene expression (*20, 25*).

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26. Cells were transfected with plasmid DNA by a combination of techniques (*11*). Monolayers of wild-type cells were trypsinized, subjected to centrifugation, and resuspended in Hepes-buffered phosphate solution containing a calcium phosphate–DNA precipitate (1 to 2 μg of plasmid DNA per 10^6 cells). After a 15-minute incubation at room temperature, the cells were plated in growth medium at 37°C. Twenty-four hours later, the cells received a 5-minute boost with 20 percent DMSO and were supplemented with growth medium. One day later the cells were exposed for an additional 24 hours to the different inducers.
27. For the CAT assay, approximately 2×10^7 cells were washed in ice-cold phosphate-buffered saline (PBS), collected by scraping them into PBS, and subjected to centrifugation. The pellet was resuspended in 0.25*M* tris-HCl and lysed by three cycles of freezing and thawing. The cells were again subjected to centrifugation (1 minute, 15,000*g*), after which the supernatants were removed and assayed for CAT activity (*10*). The assay contained, in a final volume of 100 μl, 30 to 40 μl of cell extract, 10 m*M* acetyl coenzyme A, 0.5 μCi of ^{14}C-labeled CM, and 0.25 m*M* tris-HCl (*p*H 7.8). After a 90-minute incubation at 37°C, the reaction mixture was extracted twice with an equal volume of ethyl acetate, and the organic phases were combined and evaporated to dryness. The residue was dissolved again in ethyl acetate and analyzed by thin-layer chromatography in a chloroform-methanol (95:5) system and subsequent autoradiography.
28. DNA primer (3 μg) was treated with calf intestinal alkaline phosphatase and end-labeled with adenosine [γ-^{32}P]triphosphate with the use of T4 polynucleotide kinase (specific activity, ~10^6 count/min per microgram). DNA fragments (1.5 μg) were precipitated with ethanol, suspended in 150 μl of hybridization buffer [80 percent deionized formamide, 0.4*M* NaCl, 0.01*M* pipes (*p*H 6.4)], and denatured (100°C, 5 minutes). RNA (550 μg) from TCDD-induced HAV cells was suspended in 150 μl of hybridization buffer and added to the denatured DNA fragments. The RNA-DNA mixture was denatured (85°C, 5 minutes) and allowed to hybridize overnight at 50°C. After hybridization, the reaction mixture was diluted tenfold with 0.5*M* KCl in 0.01*M* tris-HCl (*p*H 7.5) and subjected to chromatography on oligo(dT)cellulose by means of standard techniques [H. Aviv and P. Leder, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1408 (1972)]. Duplex molecules composed of the ^{32}P-labeled (+) strand of the DNA fragment and polyadenylated RNA were precipitated with ethanol and sodium acetate. The primer extension reaction mixture contained, in a total volume of 200 μl, 50 m*M* tris-HCl (*p*H 8.3), 6 m*M* $MgCl_2$, 60 m*M* NaCl, 10 m*M* dithiothreitol, 1 m*M* each of unlabeled deoxynucleotide triphosphates, 5 units of avian myeloblastosis reverse transcriptase, and 10 units of RNasin. After a 3-hour incubation at 42°C, NaOH was added to a final concentration of 0.2*M*, and the incubation was continued for another hour to degrade the RNA template. After neutralization with 1*M* HCl and addition of sodium dodecyl sulfate to 0.5 percent, the reaction mixture was extracted twice with phenol, and the ^{32}P-labeled extended DNA primers were precipitated from the aqueous phase with ethanol and sodium acetate. The products of the primer extension reaction were analyzed by electrophoresis on 8 percent polyacrylamide–7*M* urea gels with subsequent autoradiography.
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