Activation of transcription by *v-myb*: evidence for two different mechanisms

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The retroviral oncogene *v-myb* encodes a nuclear, sequence-specific DNA-binding protein. To investigate the possibility that *v-myb* encodes a transcriptional regulator, we used a transient cotransfection assay to explore the potential of *v-myb* to influence the expression of other genes. We found that expression of a chicken lysozyme promoter/CAT gene construct was activated by *v-myb* in the presence of *myb*-specific binding sites. Activation was not observed with a truncated *v-myb* protein lacking its DNA-binding domain. We also observed that expression of a hybrid human HSP70 promoter/CAT gene, lacking *myb*-specific binding sites, was activated by *v-myb*. However, in this case, the truncated *v-myb* protein, which lacked its DNA-binding domain, also activated HSP70/CAT expression, indicating that *trans*-activation of this gene construct was independent of the sequence-specific DNA-binding activity of the *v-myb* protein. These observations suggest that *v-myb* encodes a *trans*-activator and that activation of gene expression occurs by two different mechanisms, one of which involves specific binding of *v-myb* protein to the regulated gene.

[Key Words: *v-myb*; nuclear oncogenes; *trans*-activation; specific DNA binding]

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The retroviral oncogene *v-myb* encodes a 45-kD protein, p45^{*v-myb*}, which is responsible for the potential of avian myeloblastosis virus (AMV) to transform myelomonocytic cells in vivo and in vitro (Klempnauer et al. 1982, 1983; Boyle et al. 1983; Moscovici and Moscovici 1983). The molecular mechanism of transformation by *v-myb* and the basis of its cell-type specificity are not known at present. p45^{*v-myb*} is a truncated and mutated form of the 75-kD protein product of the chicken *c-myb* gene, p75^{*v-myb*} (Klempnauer et al. 1983). *c-myb* expression is restricted largely to immature hematopoietic cells (Gonda et al. 1982; Westin et al. 1982; Sheiness and Gardinier 1984) and appears to be essential for their proliferation (Gewirtz and Calabretta 1988). Both *v-myb* and *c-myb* encode nuclear DNA-binding proteins that are associated predominantly with chromatin and bind to DNA in vitro (Klempnauer and Sippel 1986). In vitro DNA binding by viral *myb* protein is specific for the sequence motif PyAAC^G/T₃G (Biedenkapp et al. 1988), suggesting that *myb* proteins function by binding to specific target regions in the genome. However, no physiological in vivo targets for *v-myb* have been identified as yet.

c-myb genes have been highly conserved during evolution, particularly within the region that encodes their DNA-binding domain (Klempnauer and Sippel 1987; Peters et al. 1987). Genes encoding similar domains have been identified in vertebrates (Gonda and Bishop 1983; Gonda et al. 1985; Bender and Kuehl 1986; Gerondakis and Bishop 1986; Rosson and Reddy 1986; Slamon et al. 1986), in insects (Katzen et al. 1985; Peters et al. 1987), and in plants (Paz-Ares et al. 1987). The *myb*-related c1-

gene of *Zea mays* (Paz-Ares et al. 1987) has been defined genetically as a regulator of genes, which are involved in anthocyanin biosynthesis. This suggests that *myb* genes encode a family of regulatory, sequence-specific DNA-binding proteins, which control the expression of certain target genes.

To explore the regulatory potential of *v-myb*, we used artificial gene constructs as model genes, cotransfected them with *v-myb* expression vectors into eukaryotic cells, and investigated the effect of *v-myb* on the activity of these model genes. Our results show that *v-myb* activates gene expression by two different mechanisms. Expression of hybrid HSP70/CAT (chloramphenicol acetyltransferase) gene constructs is activated by *v-myb* in a DNA-binding-independent fashion. In contrast, a gene construct containing *myb*-specific DNA-binding sites upstream of the chicken lysozyme promoter is activated by *v-myb* in a DNA-binding-dependent fashion. This latter observation provides the first evidence that *v-myb* protein binds directly to target genes to stimulate their expression.

Results

v-myb encodes a *trans*-activating activity

To investigate the regulatory potential of *v-myb*, we cotransfected expression vectors for *v-myb* protein with hybrid gene constructs, containing indicator genes (coding for CAT or luciferase), fused to promoter regions of interest. The structure of the *v-myb* expression vectors is outlined in Figure 1A. After transfection of the

Figure 1. Expression of v-myb. (A) v-myb expression vectors used in this study. (Top) The general structure of the expression vectors and the spliced mRNA encoding v-myb. The ATG codon indicated is the gag gene ATG that provides the translational start for the v-myb protein in AMV and in the expression vectors (Klempnauer et al. 1983). pVM116 encodes a full-length v-myb protein. pVM111 contains a 4-bp insert at the NcoI site (N) close to the start of the v-myb-coding region. pVM130 encodes an amino-terminally truncated v-myb protein lacking sequences 5' to the EcoRI (E) site in v-myb. Other restriction sites are BamHI (Ba), BglII (Bg), KpnI (K), and XbaI (X). (Only relevant restriction sites are shown). (B) Western blot analysis of quail fibroblasts transfected with 5 μ g each of the vectors described in A. (C) Western blot analysis of subcellular fractions of quail fibroblasts transfected with 5 μ g each of pVM116 or pVM130. (T) Unfractionated cells; (C) cytoplasmic fraction; (N) nuclear fraction. In B and C, myb proteins were visualized by immunostaining with the myb-specific mAb myb2-2 (Evan et al. 1984). Full-length and truncated v-myb proteins are marked by open and closed arrowheads, respectively.

quail fibroblast cell line QT6 (Moscovici et al. 1977), myb protein could be detected by Western blotting with the myb-specific mAb myb2-2 (Fig. 1B; Evan et al. 1984). A control vector, containing a frameshift mutation in the v-myb-coding region close to its 5' end, did not yield detectable myb protein after transfection. Figure 1C shows that even after deletion of the amino-terminal region harboring the v-myb DNA-binding domain, most of the expressed myb protein was transported to the cell nucleus, suggesting that a nuclear localization signal resides in the carboxy-terminal portion of the v-myb protein. This observation is in contrast to a previous report, which suggested the existence of a nuclear localization signal in the amino-terminal, highly conserved region of the v-myb protein by showing that deletion of this region abolished nuclear localization (Ibanez and Lipsick 1988). Presently, we have no explanation for this discrepancy.

In initial experiments we tested the influence of v-myb on the expression of a panel of different gene constructs, whose structures are outlined in Figure 2A. Representative transfections, routinely performed in duplicate, are illustrated in Figure 2B–G. There was either no influence of v-myb on the expression of the transfected test gene (e.g., SV40-CAT, SV40-luciferase, or chicken LYSCAT) or v-myb stimulated the expression of the reporter gene (human and *Drosophila* HSP70/CAT). Activation HSP70/CAT expression is unlikely to be a stress response (caused, e.g., by the transfection procedure), because deletion of the heat shock response element, centered ~100 bp upstream of the transcriptional start site in the human HSP70 promoter and mediating stress re-

sponse (Wu et al. 1986), did not abolish v-myb dependent activation (Fig. 2F).

To demonstrate that the v-myb-induced activation of HSP70/CAT expression was due to an increase in the level of transcripts initiated at the HSP70 promoter, we performed nuclease S1 mapping analyses of RNA isolated from transfected cells. As illustrated in Figure 3, the amount of mRNA changed in parallel to the CAT activity. Thus, v-myb appeared to act on the level of transcription or to affect RNA stability or transport.

The experiments described thus far have been performed with the quail fibroblast cell line QT6. These cells do not express endogenous c-myb and are transfected efficiently. However, like chicken fibroblasts, quail fibroblasts cannot be transformed by AMV. Therefore, we were concerned that the trans-activating activity of v-myb observed in QT6 cells did not mirror myb function in a myeloid cell transformed by v-myb. We repeated some of the transfection experiments using MC29-transformed chicken macrophages, which can be transformed by v-myb (Ness et al. 1987). Like QT6 cells, HBCI cells do not express detectable endogenous c-myb protein; however, transfection of HBCI cells was ~10-fold less efficient when compared with QT6 cells. The experiment illustrated in Figure 4 showed that v-myb activated HSP70/CAT expression to a similar extent in both HBCI and QT6 cells. Gene constructs that were not affected by v-myb in QT6 cells also were not affected when transfected into HBCI cells (data not shown). These experiments suggested that the trans-activating potential of v-myb is similar in a target and a nontarget cell.

Figure 2. Trans-activation by *v-myb*. (A) Schematic illustration of reporter plasmids used in this study. Numbers below the constructs refer to positions relative to transcriptional start sites, marked by arrows. pSV2CAT and pSV2B2 contain the SV40 early promoter and enhancer fused to the CAT- or luciferase-coding regions, respectively. pDHSP70CAT contains the promoter region of a *Drosophila* HSP70 gene (Amin et al. 1985). Other HSP70/CAT plasmids contain the promoter region of the human HSP70 gene (Kaddurah-Daouk et al. 1987). pLYSCAT constructs contain the promoter region of the chicken lysozyme gene (Theisen et al. 1986). (534) A sequence to which *v-myb* protein binds in vitro (Biedenkapp et al. 1988). (B–G) Different reporter plasmids (1 μ g in B and C; 5 μ g in D–G) were cotransfected with pVM116 (+) or pVM111 (–) (5 μ g of each) and with 2 μ g each of pSV2B2 (B, and D–G) or pSV2CAT (C) into quail fibroblasts. Cells were harvested and analyzed 24 hr after transfection. Numbers below lanes indicate percent conversion of chloramphenicol to acetylated derivatives. In (C) Relative luciferase activities are indicated by the size of the columns. Each lane represents an individual cell culture dish. Transfection efficiencies were normalized by analyzing the activities of cotransfected pSV2B2 (B, and D–G) and pSV2CAT (C) gene constructs.

DNA binding of *v-myb* protein is not required for activation of HSP70/CAT expression

Previously, we have shown that *v-myb* encodes a sequence-specific DNA-binding protein, recognizing the sequence motif PyAAC^G/_TG (Biedenkapp et al. 1988). DNA-binding studies with the HSP70 promoter region contained in plasmid pHSP70CAT/-120 have not yielded any evidence for specific binding of *v-myb* protein to the HSP70 promoter [data not shown]. This lack of specific binding is consistent with a lack of *myb* recognition motifs from the HSP70 promoter. Therefore, it was interesting to investigate whether *v-myb*-induced activation of HSP70/CAT expression is dependent on the sequence-specific DNA-binding function of the *v-myb* protein. To address this question, we cotransfected the HSP70/CAT gene construct with a *v-myb* expression vector (pVM130), encoding an amino-terminally truncated version of the *v-myb* protein. The truncated protein lacks the highly conserved DNA-binding domain and does not bind to DNA (Klempnauer and Sippel 1987; Peters et al. 1987); as shown in Figure 1,

this protein is made in similar quantities as the full-length *v-myb* protein and is present in the nucleus. The experiment illustrated in Figure 5 demonstrated that the amino-terminally truncated protein *trans*-activated the HSP70/CAT gene construct quite efficiently when compared to the full-length *v-myb* protein, indicating that DNA binding of *v-myb* protein was not required for activation of HSP70/CAT expression.

A *v-myb*-binding site confers *v-myb* responsiveness to a reporter gene

The lack of a requirement for specific DNA binding in activation of HSP70/CAT expression by *v-myb* raised the question of whether specific DNA binding by *v-myb* protein to its cognate binding sites plays a role in gene regulation at all. Previously, we isolated *myb*-specific in vitro DNA-binding sites from the chicken genome (Biedenkapp et al. 1988). The availability of these sequences allowed us to test whether the presence of *myb*-specific binding sites affects the *myb*-responsiveness of reporter genes. One of the *myb*-binding sequences, designated

Figure 3. Nuclease S1 mapping of HSP70/CAT transcripts. (A) CAT assay of quail fibroblasts transfected with pHSP70CAT/–120 (3 μ g), pSV2B2 (2 μ g), and pVM116 (+) or pVM111 (–) (5 μ g each). Cells were harvested 24 hr after transfection. Transfection efficiencies were normalized by analyzing the activity of the cotransfected pSV2B2 gene construct. Numbers below lanes indicate percent conversion of chloramphenicol to acetylated derivatives. Each lane represents two separately transfected cell culture dishes pooled at the time they were harvested. (B) Nuclease S1 mapping of RNA isolated from the transfected cells shown in A. The upper band is the full-length probe fragment, and the lower band is the promoter-specific S1-resistant fragment. The mapping strategy is outlined at the bottom. (Right) Sizes and positions of length markers.

534 (Biedenkapp et al. 1988), was cloned upstream of the chicken lysozyme promoter region in the pLYSCAT1000 gene construct. As described before, the 534 sequence element contains several *v-myb* consensus binding motifs recognized by *v-myb* protein in vitro (Biedenkapp et al. 1988). Expression of the original gene construct lacking binding sites was affected only slightly by *v-myb* (Figs. 2G and 6B). However, in the presence of the *v-myb*-binding sites (p534LYSCAT1000-2), CAT expression was activated by *v-myb* between 10- and 20-fold, demonstrating that the *myb*-binding sites conferred *myb* responsiveness to the chicken lysozyme promoter/CAT gene construct. Transfection of HBCI cells instead of QT6 cells showed a similar activation of p534LYSCAT1000-2 expression, suggesting again that the *trans*-activating activity of *v-myb* is similar in both cell types (data not shown). Analysis of RNA isolated from transfected cells showed that the *myb*-induced activation of expression was accompanied by increased abundance of transcripts initiated at the chicken lysozyme promoter (Fig. 6C). If, as we assume, *v-myb* protein activates p534LYSCAT1000-2 expression by interacting with its binding sites, we would expect a truncated

v-myb protein, lacking its DNA-binding domain, to be inactive in stimulating expression of the p534LYSCAT1000-2 gene construct. To demonstrate that the *myb*-induced activation of p534LYSCAT1000-2 expression required the *v-myb* DNA-binding domain and therefore was qualitatively different from activation of HSP70/CAT expression, we tested the ability of the amino-terminally truncated *v-myb* protein to activate expression of the p534LYSCAT1000-2 gene construct. Figure 6D shows that the truncated *v-myb* protein, lacking its DNA-binding domain, did not stimulate expression of p534LYSCAT1000-2. This strongly suggested that activation of expression of this gene construct was due to specific binding of *v-myb* protein to its cognate binding sites.

Discussion

The retroviral oncogene *v-myb* encodes a nuclear, sequence-specific DNA-binding protein (Biedenkapp et al. 1988); therefore it is possible that the *v-myb* protein directly interacts with certain target genes to regulate their expression. This hypothesis is consistent with the finding that the *myb*-related *c1* gene of *Zea mays* is a regulatory gene (Paz-Ares et al. 1987) and that one of the genes regulated by *c1*, the maize *a1* gene, is associated with *myb*-binding sites (Biedenkapp et al. 1988; K.-H. Klempnauer, H. Arnold, and H. Biedenkapp, unpubl.). Because target genes for *v-myb* have not been identified yet, we explored the regulatory potential of *v-myb* by an indirect strategy, namely by testing the effect of *v-myb* on the expression of various hybrid gene constructs.

The results presented here show that as with other nuclear oncogenes, such as *jun* (Bohmann et al. 1987;

Figure 4. Activation of human HSP70/CAT expression in HBCI cells. HBCI cells were transfected with pHSP70CAT/–120 (3 μ g), pSV2B2 (2 μ g), and pVM116 (+) or pVM111 (–) (5 μ g each). Cells were harvested 24 hr later. Transfection efficiencies were normalized by analyzing the activity of the cotransfected pSV2B2 gene construct. Numbers indicate percent conversion of chloramphenicol to acetylated derivatives. Each lane represents a single transfected cell culture dish.

Figure 5. Activation of human HSP70/CAT expression by a truncated *v-myb* protein. (A) CAT assays of quail fibroblasts transfected with pHSP70CAT/−120 (3 μ g), pSV2B2 (2 μ g), and the indicated *myb* expression vectors (5 μ g each). Cells were harvested and analyzed 24 hr after transfection. Transfection efficiencies were normalized by analyzing the activity of the cotransfected pSV2B2 gene construct. Numbers indicate percent conversion of chloramphenicol to acetylated derivatives. Each lane represents two separately transfected cell culture dishes pooled when they were harvested. (B) Nuclease S1 mapping analysis of RNA isolated from the transfected cells shown in A. The upper and lower bands are the full-length probe fragment and the promoter-specific S1-resistant fragment, respectively. The mapping strategy is the same as in Fig. 3.

Angel et al. 1988), *fos* (Setoyama et al. 1986; Chiu et al. 1988; Lucibello et al. 1988; Sassone-Corsi et al. 1988), and *myc* (Kingston et al. 1984; Kaddurah-Daouk et al. 1987), *v-myb* encodes a *trans*-activating activity. Therefore, our findings are consistent with the possibility that the *v-myb* protein is a transcriptional regulator and that oncogenic transformation by *v-myb* may be brought about by deregulation of certain *v-myb* target genes.

While this manuscript was in preparation, Nishina et al. (1989) demonstrated *trans*-activation by mouse *c-myb*. Our results extend the findings of these investigators in two directions. First, as discussed below, we show that specific DNA binding might be involved in *trans*-activation by *myb*. Second, we demonstrated a *trans*-activating activity of *v-myb*. Thus, it appears that the *trans*-activation function of *myb* has been preserved during generation of *v-myb*, suggesting that this activity is required for transformation.

Activation of gene expression by *v-myb* occurs by two different mechanisms. Our results show that specific *in vitro* DNA-binding sites for *v-myb* protein confer *myb* responsiveness to a chicken lysozyme promoter/CAT gene construct. In addition to requiring the presence of *myb*-binding sites, activation of expression depends on the presence of the DNA-binding domain in the *v-myb*

protein. The simplest explanation of these results is that the *v-myb* protein *in vivo* interacts with its cognate binding sites and, in doing so, increases the expression of the bound gene construct. These observations represent the first evidence that the *v-myb* protein *in vivo* recognizes specific binding sites and that *v-myb* can regulate expression of a gene by directly binding to it via specific DNA-binding sites. We do not know whether the *v-myb*-binding site we used is recognized efficiently *in vivo* or whether a precise arrangement of *myb*-binding sites and other promoter elements is required for optimal activation by *v-myb*. These uncertainties may limit the degree of activation observed.

In contrast to the binding-site-dependent mode of activation, stimulation of HSP70/CAT expression by *v-myb* does not require the sequence-specific DNA-binding function of *v-myb*. This conclusion is based on the observation that a truncated *v-myb* protein, lacking the DNA-binding domain, is still active as a *trans*-activator of the HSP70/CAT gene. Thus, *v-myb* might be capable of activating expression of certain genes in a DNA-binding-independent mode. The potential to affect gene expression by two different mechanisms has interesting implications for the regulation of the *myb trans*-activating function. If both activating activities are regulated separately, *myb* may be capable of controlling expression of different target genes independently of one another.

It is interesting that *v-myb* functions as a *trans*-activator in a cell type that cannot be transformed by *v-myb*. We have no proof at present that the *trans*-activating activity of *v-myb* is essential for cell transformation. If it is, however, our results suggest that the target cell specificity of *v-myb* is unlikely to be due to production of nonfunctional *v-myb* protein in nontarget cells. It is perhaps more likely that cellular targets, such as DNA sequences with which the protein must interact for transformation to occur, are not accessible in nontarget cells. That *v-myb* protein interacts with transfected binding sites in quail fibroblasts is not inconsistent with this view, because the accessibility of transfected sequences to the protein might be very different from that of the resident sequences.

Our experiments do not directly address the nature of the mechanisms by which *v-myb* activates gene expression. Because the *myb* responsiveness of the lysozyme promoter/CAT gene construct was affected by sequences lying upstream of the mRNA start site and are therefore not present in the RNA, it is likely that the *v-myb* activates expression of this gene construct by increasing its transcription, rather than by affecting transport or half-life of the RNA. In case of the *myb*-binding site independent mode of activation, it is possible that *v-myb* acts on the level of transcription (e.g., by influencing the activities of certain transcription factors directly or indirectly). However, it would also be consistent with our data if *v-myb* activated HSP70/CAT expression by affecting transport or half-life of the HSP70/CAT RNA. Identification of HSP70/CAT sequences that confer *myb* responsiveness should provide a means of resolving this question.

Figure 6. Effect of *v-myb*-binding sites on activation of chicken *LSYCAT* expression. (A) Location of *myb* consensus binding motifs (arrowheads) in the chicken genomic sequence 534 (Biedenkapp et al. 1988). (B and D). CAT assays of quail fibroblasts, transfected with the indicated DNAs (5 μ g of each) and with 2 μ g of pSV2B2 to monitor transfection efficiencies. Each lane represents a single transfected cell culture dish. Numbers indicate percent conversion of chloramphenicol to acetylated derivatives. Transfection efficiencies were normalized by analyzing the activity of the cotransfected pSV2B2 gene construct. (C) Ribonuclease protection analysis of RNA isolated from quail fibroblasts transfected as indicated. Each lane represents two separately transfected cell culture dishes pooled at the time they were harvested. The arrowhead indicates to the RNase-resistant promoter-specific fragment. The band below presumably results from protection of a less than full-length probe molecule. The mapping strategy is outlined at bottom. In all experiments, cells were harvested 24 hr after transfection.

Materials and methods

Cells and antibodies

QT6 is a line of chemically transformed quail fibroblasts (Moscovici et al. 1977). HBC1 is a line of MC29-transformed chicken macrophages, kindly provided by H. Beug. All cells were grown in Iscove's modified Dulbecco's minimal Eagle's medium (DMEM), supplemented with 2% chicken serum and 8% fetal calf serum. The *myb*-specific mAb *myb2-2* has been described previously (Evan et al. 1984).

Western blotting

Western blotting was performed as described previously (Klempnauer et al. 1986).

Plasmids

An expression vector for full-length *v-myb* protein (pVM116) was constructed as follows. A 2.3-kb *Xba*I–*Bgl*III fragment, encompassing two tandem copies of the MAV-2 long terminal repeat (LTR) and adjoining *gag* sequences, was taken from a cloned copy of a myeloblastosis-associated virus (G. Ramsay,

D. Westaway, K.-H. Klempnauer, H.E. Varmus, and J.M. Bishop, unpubl.) and cloned upstream of a 2.5-kb *Bgl*II–*Kpn*I fragment of the clone pVM2 (Klempnauer et al. 1982), containing the *v-myb* gene and flanking viral sequences including the downstream LTR of an integrated AMV. A 0.85-kb *Bam*HI–*Bgl*III fragment of plasmid pKm18 (Beck et al. 1982), carrying the neomycin resistance gene, was inserted into the single *Bgl*II site upstream of the *v-myb* gene. In cells carrying this vector, *v-myb* is expressed from a spliced mRNA, as in the original AMV. Translation of the *v-myb* protein starts at the *gag* ATG; thus, the protein encoded by pVM116 is identical to authentic p45^{*v-myb*} encoded by AMV (Klempnauer et al. 1982). pVM111 was constructed by filling in the 5' overhanging ends of a *Nco*I site located at the 5' end of the *v-myb*-coding region, thereby creating a frameshift. To obtain pVM130, a *Hin*II site, GAATC, located exactly at the start of the *v-myb*-coding region (Klempnauer et al. 1982), was converted to an *Eco*RI site, GAATTC, and fused with the single *Eco*RI site within *v-myb*. The protein encoded by pVM130 lacks the amino-terminally located *v-myb* DNA-binding domain. Most reporter plasmids used in *trans*-activation experiments have been described previously (see legend to Fig. 2). pSV2B2, a derivative of pSV2CAT containing the firefly luciferase gene (De Wet et al. 1987) instead of the CAT gene, was a gift of C. Peters.

DNA transfection and CAT and luciferase assays

DNA was transfected into QT6 or HBCI cells by the calcium phosphate coprecipitation method [Graham and van der Eb 1973], as modified by Theisen et al. (1986). Cell extracts to be used for enzyme assays were prepared by suspending the cells in 0.1 M potassium phosphate (pH 7.8) and 1 mM dithioerythrol, freeze-thawing them three times, and spinning down debris. CAT and luciferase assays were performed as described [Theisen et al. 1986; De Wet et al. 1987]. CAT activities were quantitated by measuring the amounts of the ^{14}C -labeled chloramphenicol substrate and the acetylated products in a scintillation counter.

RNA analyses

Total cellular RNA was prepared as follows. Transfected cells were washed in PBS and lysed in 4 M guanidinium thiocyanate, 0.5% sarcosyl, 25 mM sodium citrate (pH 7.0), and 1 mM β -mercaptoethanol. DNA was sheared by passing the lysate through a syringe until it was no longer viscous. Then the lysate was layered on the top of a cushion composed of 5.7 M CsCl, 50 mM sodium acetate (pH 5.4), and 50 mM EDTA and centrifuged in an SW 60 rotor at 36,000 rpm at 20°C for 16 hr. The pelleted RNA was dissolved in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA, ethanol-precipitated three times, and then used directly for S1 or ribonuclease protection studies. S1 mapping was performed as described [Theisen et al. 1986]. The probe used for S1 mapping was a 270-bp *Bgl*II–*Hind*III fragment of plasmid pHSP70CAT/–120. This fragment encompasses the HSP70 promoter region from –120 to +150 bp [Kaddurah-Daouk et al. 1987]. To generate a radiolabeled probe suitable for ribonuclease protection studies, a 330-bp *Cla*I–*Pvu*II fragment of plasmid pLYSCAT1000 (encompassing the 5' end of the CAT gene and chicken lysozyme promoter sequences up to –161 bp; Theisen et al. 1986) was cloned into the vector pBlue-scriptKSM13–. Then the DNA of this plasmid was linearized with *Cla*I and transcribed with T7 RNA polymerase. Hybridization of the probe to RNA and ribonuclease digestion were performed by published procedures [Melton et al. 1984].

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