differential use of the core promoter by the two cell lines, an explanation that would be consistent with our observation that the enhancer in the 88-bp core fragment is more active in HuH6 cells than that in Hep G2 cells. Since Hep G2 and HuH6 cells may represent hepatocytes arrested at different stages of differentiation, as indicated by the fact that they express different sets of liver-specific proteins (*14*), this core enhancer may be developmentally regulated. Honigwachs *et al.* have recently shown that a similar core fragment failed to activate CAT expression in hepSK cells, a de-differentiated human liver line (*15*). Together, these results raise the interesting possibility that the absence of HBV replication and core gene expression observed in advanced hepatocellular carcinoma could be due to the decrease of the core enhancer activity in proportion to the de-differentiation of the infected hepatocytes.

It has been suggested that the previously described HBV enhancer may determine in part the liver-specific expression of the HBV pregenomic RNA (*16*). The results presented here indicate that the previously described enhancer does indeed function in human hepatoma cells, but it also stimulates CAT expression in nonhepatic cells. In contrast, activity of the enhancer located in the 88-bp core promoter region is restricted to human hepatoma cells. Since the 88-bp fragment by itself is sufficient to direct efficient CAT expression in hepatoma cells, it obviously also contains a functional promoter which, together with the tissue-specific enhancer, may account for the liver specificity of HBV gene expression. These studies of the HBV core promoter have demonstrated that a liver-specific enhancer activates expression from the core promoter in highly differentiated hepatoma cells. Deletion analysis of the core promoter region also suggests that a negative element in the core promoter may account for the extremely low activity of this promoter in nonhepatic cells. Such a combination of positive and negative regulatory effect may attribute to the highly liver-specific expression of the HBV 3.5-kb pregenomic RNA. The discovery of these regulatory signals in HBV should allow more informative studies on the regulation of HBV expression and replication in infected cells and the role of HBV in the development of hepatic malignancies.

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Initiation by Yeast RNA Polymerase II at the Adenoviral Major Late Promoter in Vitro

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Transcription of the yeast *CYC1* promoter fused to a sequence lacking guanosine residues provided a rapid, sensitive assay of initiation by RNA polymerase II in yeast extracts. Initiation was enhanced by yeast and mammalian activator proteins. The adenoviral major late promoter fused to the G-minus sequence was transcribed in yeast extracts with an efficiency comparable to that observed in HeLa extracts, showing that promoters as well as transcription factors are functionally interchangeable across species. Initiation occurred at different sites, approximately 30 and 63 to 69 base pairs downstream of the TATA element of the adenoviral promoter in HeLa and yeast extracts, respectively, distances characteristic of initiation in the two systems in vivo. A component of the transcription system and not the promoter sequence determines the distance to the initiation site.

MANY ASPECTS OF TRANSCRIPTION by RNA polymerase II are conserved between yeast and higher eukaryotes. There is extensive amino acid sequence similarity among the largest subunits of the yeast, *Drosophila*, and mammalian polymerases (*1*). Other components of the transcription apparatus, such as TATA-binding and enhancer-binding factors, are functionally interchangeable between yeast and mammalian systems (*2–5*). There are, nonetheless, significant differences between the two systems. TATA elements are located from 40 to 120 or more base pairs upstream of the initiation site of a yeast promoter, and where these elements occur, they are absolutely required (*6*). By contrast, the distance between TATA elements and transcription start sites is smaller, 25 to 30 bp, and more uniform among mammalian promoters; in some cases, deletion of a TATA element does not reduce the frequency of initiation but rather impairs the precision of the process (*7*). It has been shown that if a yeast TATA-binding protein is substituted for the corresponding factor from HeLa cells, initiation still occurs in the manner characteristic of mammalian systems (*2, 3*). This implies that a factor other than the TATA-binding protein measures out the distance to the initiation site, or that the promoter sequence dictates the location of the site. Here we report on studies with a yeast transcription system that rule out the latter possibility and that may help identify the protein factor that locates the initiation site.

The recent development of a yeast RNA polymerase II transcription system (*8*) depended on hybridization with an RNA probe to reveal accurately initiated transcripts against a high background of nonspecific reaction. This method of detection is time-consuming and laborious. We tried a number of alternatives, such as run-off and primer extension assays, and had most success with an approach involving transcription of a template devoid of G residues (*9*). A form of the yeast *CYC1* promoter was

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prepared (*10*) with a G residue at position −35 (with respect to the translation start site at +1) replaced by a C, followed by fusion at this residue to the 377-bp G-minus cassette of Sawadogo and Roeder (*9*). Accurately initiated transcripts extending through the cassette were expected to contain a G-minus stretch of ~390 residues. Background due to transcription of other sequences was suppressed by the use of the chain terminator 3′-*O*-methyl guanosine triphosphate (GTP), and extraneous transcripts were destroyed by digestion with T1 ribonuclease (RNase) (*9*). G-minus transcripts were revealed and quantitated by deproteinization and gel electrophoresis. Transcripts of approximately 350 and 370 residues were obtained (Fig. 1). The initiation reaction appeared to be authentic on the basis of the following observations.

First, a promoter was absolutely required. No G-minus transcripts were obtained from the G-minus cassette alone (Fig. 2, right panel). This argues against the possibility that transcription started upstream of the promoter and read through the cassette, with transcripts of the expected length resulting entirely from T1 RNase digestion. Further evidence that the observed transcripts were not artifacts of readthrough transcription came from experiments in which the 3′-*O*-methyl GTP chain terminator was omitted from the reaction (*11*). An additional transcript appeared, presumably the product of readthrough transcription, about 30 residues longer than the pair of transcripts seen in the presence of the chain terminator, and thus clearly distinguished from these transcripts.

Second, no G-minus transcripts were obtained in the presence of α-amanitin at 10 μg/ml, a concentration known to inhibit RNA polymerase II but not polymerases I and III in vitro (*12*).

Third, the sites of initiation in vitro corresponded closely with those used in vivo.

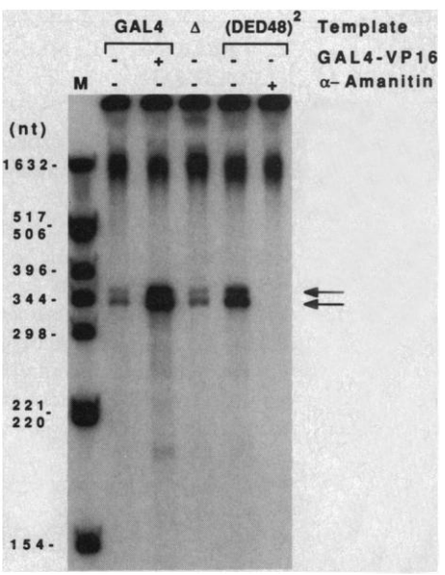

Fig. 1. Transcription of *CYC1*/G-minus templates in a yeast nuclear extract, and effects of activator proteins. Templates pGAL4CG−, pΔCG−, and p(DED48)²CG− (*10*) are abbreviated GAL4, Δ, and (DED48)², respectively. Reaction mixtures (30 μl) contained 0.1 μg of template, yeast nuclear extract (90 μg of protein) prepared as described (*14*), 0.2 unit of Inhibit-ACE (5′–3′), and 0.5 μCi of [α-^{32}P]uridine triphosphate (UTP) (600 Ci/mmol) in 50 m*M* Hepes, *p*H 7.3, 70 m*M* KOAc, 5 m*M* MgOAc, 5 m*M* $MgSO_4$, 2.5 m*M* dithiothreitol, 5 m*M* EGTA, 4 m*M* phosphoenolpyruvate, 0.4 m*M* each of adenosine triphosphate (ATP) and cytidine triphosphate (CTP), and 10% glycerol. Reactions were supplemented, where indicated, with 20 ng of GAL4-VP16 (*15*), incubated with the template for 5 min at 4°C before the addition of nuclear extract, or with α-amanitin (10 μg/ml). Reactions were allowed to proceed for 1 hour at 20°C, followed by treatment with RNase T1 (10 units in 200 μl of 10 m*M* tris-HCl, *p*H 7.5, 300 m*M* NaCl, and 5 m*M* EDTA) for 10 min at room temperature, treatment with 12 μl of 10% SDS and 100 μg of proteinase K for 20 min at 30°C, and ethanol precipitation. Transcripts were analyzed by electrophoresis in a 7% polyacrylamide–7*M* urea gel in TBE buffer (*19*) and autoradiography. Arrows indicate bands due to G-minus transcripts. Size markers in the leftmost lane were Hin fI fragments of pBR322; nt, nucleotides.

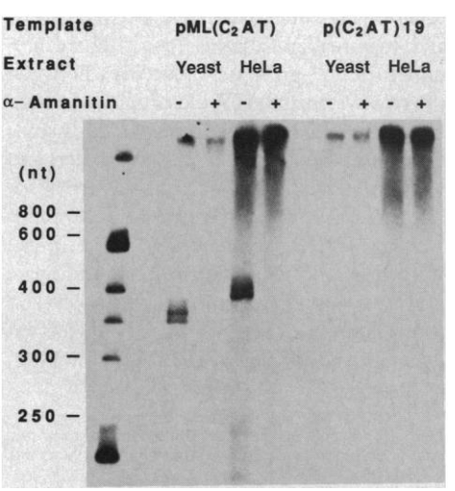

Fig. 2. Transcription of an adenoviral major late promoter/G-minus template in yeast and HeLa nuclear extracts. Transcription in yeast extract was as in Fig. 1. Transcription in HeLa extract was as described (*20*) for 1 hour at 30°C in 40 μl of 50 m*M* Hepes, *p*H 7.9, 6 m*M* $MgCl_2$, 40 m*M* KCl, 1 m*M* dithiothreitol, 0.1 m*M* EDTA, 10% glycerol, protein at 1 mg/ml, with template, nucleotide, Inhibit-ACE, and phosphoenolpyruvate concentrations as in Fig. 1. Templates (*9*) were pML-(C_2AT), containing the adenoviral major late promoter (residues −400 to +10) upstream of the 377-bp G-minus cassette, and p(C_2AT)19, containing only the G-minus cassette. Concentrations of α-amanitin were 10 and 0.5 μg/ml in reactions with yeast and HeLa extracts, respectively. Transcripts were isolated and analyzed as for Fig. 1. Size markers in the leftmost lane were as in Fig. 1; nt, nucleotides.

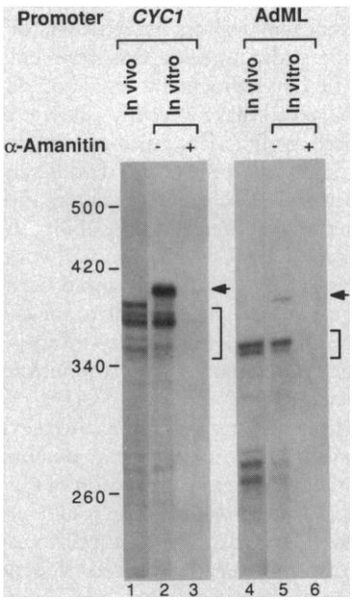

Fig. 3. RNase protection mapping of transcripts of *CYC1*/G-minus and adenoviral major late promoter/G-minus templates synthesized in yeast in vivo and in yeast nuclear extract. Transcripts synthesized in vivo were isolated from *Saccharomyces cerevisiae* strain 5C harboring either pCZ3GALCG− [three GAL4-binding sites in front of the *CYC1*/G-minus cassette fusion promoter (lane 1)] or pCZ3GALMLG− [same as preceding except with adenoviral major late promoter fusion (lane 4)] grown in the presence of galactose as described (*15*). Transcripts were synthesized in vitro from pGAL4CG− (lanes 2 and 3) or from pMLG− (lanes 5 and 6) as described in Fig. 1, except scaled up to 100 μl and with 0.4 m*M* unlabeled UTP instead of [^{32}P]UTP. RNase protection mapping was as described (*8*), with RNA probes made from either pSPCG− (lanes 1 to 3) or pSPMLG− (lanes 4 to 6) by cleavage with Eco RI and transcription with SP6 RNA polymerase. Lanes 1 and 4 were exposed five times as long as the others. The arrows indicate readthrough transcripts of the promoter and the G-minus cassette (for both the *CYC1* promoter and the adenovirus major late promoter templates) that have been shown to be suppressed by addition of the chain terminator, 3′-*O*-methyl GTP (see text). Plasmids were constructed as follows: pMLG−, insertion between the Eco RI and Bam HI sites of pUC18 of a synthetic oligonucleotide containing the minimal adenoviral major late promoter (residues −50 to +3 with respect to the transcription start site, with an Eco RI sticky end at −50 and a blunt end at +3) and an Alu I–Bam HI fragment of p(C_2AT)19 containing the G-minus cassette; pCZ3GALCG− and pCZ3GALMLG−, replacement of the Eco RI–Bam HI fragment of pCZ3GAL (*15*) with the Eco RI–Bam HI fragments of pGAL4CG− and pMLG−, respectively; pSPCG− and pSPMLG−, insertion into pSP64 of the Eco RI–Bam HI fragments of pGAL4CG− and pMLG−, respectively.

Transcripts of the *CYC1*/G-minus cassette fusion obtained either from the in vitro system or after transformation into yeast were annealed to complementary ^{32}P-labeled RNA probes, digested with RNase, and analyzed by gel electrophoresis (Fig. 3, left panel). The major clusters of transcription start sites mapped in this way were located from 5 bp upstream to 30 bp downstream of the beginning of the G-minus cassette (bracket, left panel of Fig. 3). These locations of start sites were consistent with the lengths of G-minus transcripts (Fig. 1). The pattern of start sites in vitro was identical to that in vivo (with the exception of the uppermost transcription signal in Fig. 3, marked with an arrow, which was not obtained reproducibly, and which probably derived from readthrough transcription of the promoter and cassette). The only difference between the patterns of initiation in vitro and in vivo was in the relative frequencies of the various starts, much as was previously found for a *CYC1*–lac Z fusion gene (*8*).

Finally, initiation was responsive to activator proteins, both a mammalian activator, consisting of the DNA-binding domain of GAL4 protein (residues 1 to 147) fused to the activation domain of herpes VP16 (*13*), and a yeast activator, whose DNA binding site is a thymidine-rich element (*14*). For these studies, a single GAL4-binding site (with the sequence of the high-affinity binding site near the *GAL10* gene) or a pair of T-rich elements (48-bp synthetic oligonucleotides with the sequence of the T-rich element from upstream of the *DED1* gene) were placed 24 bp upstream of the TATA element of the *CYC1* promoter fused to the G-minus cassette [giving pGAL4CG− and p(DED48)^{2}CG− (*10*)]. With GAL4-VP16 protein added to the reaction, the template with a GAL4-binding site gave nine times as many G-minus transcripts as were obtained in the absence of the activator protein or with a template lacking a GAL4-binding site (pΔCG−). This degree of stimulation is comparable to that measured by the hybridization assay (*15*) (about a tenfold stimulation for a template with a single GAL4-binding site). The template with a pair of T-rich elements gave five times as many G-minus transcripts as the control template lacking such elements, within the range of previous results from the hybridization assay (*14*).

Despite the differences in structure between yeast and mammalian promoters noted above, yeast extracts were capable of initiation at the adenoviral major late promoter (Fig. 2). The efficiency, judged from the intensity of the bands due to G-minus transcripts, was only slightly less than that obtained with HeLa nuclear extracts. The transcripts were, however, shorter in the yeast than in the HeLa system, indicating that transcription started further downstream of the TATA element. This downstream shift was not an anomaly of the in vitro system, since initiation occurred at the same sites in yeast in vivo (Fig. 3, right panel; again, as discussed above, the uppermost band, indicated by an arrow, was not consistently observed, and was probably a product of readthrough transcription). Initiation sites were determined more precisely by primer extension with reverse transcriptase (Fig. 4). Initiation occurred in yeast extracts at positions +34 and +40 with respect to the start site in mammalian cells at +1, whereas initiation in HeLa extracts was at position +1. The initiation sites used in yeast extracts (and in yeast in vivo) therefore lay within the G-minus cassette.

The aspects of transcription initiation described here are consistent with previous studies of the sequence requirements for initiation in yeast. It was previously found that both the TATA element and the sequence of the initiation site are important. The TATA element appears to define a window from about 40 to 120 bp downstream within which initiation can occur (*16*). The precise location of the initiation site then depends on the sequence surrounding the site, and this is not very stringently defined, resulting often in multiple transcription starts (*16, 17*). A synthetic TATA element of the form TATAAA is fully effective in vivo (*18*), and the TATA element of the adenoviral major late promoter contains this sequence, so it is not surprising, in retrospect, that the adenoviral promoter functions in the yeast system. The transcription start sites in the yeast system, ranging from 63 bp downstream of the TATA element of the adenoviral promoter (distance measured from the first T of the TATA element to the 5′-most initiation site) to 119 bp downstream of the *CYC1* TATA element (distance to the 3′-most initiation site), all lie within the allowed window and represent sequences in the *CYC1* promoter or G-minus cassette that are favorable for initiation.

The capacity of yeast cells and extracts to initiate transcription at the adenoviral major late promoter rules out a possible requirement for special sequences and corresponding protein factors unique to yeast. The high efficiency of initiation in yeast extracts indicates that difficulties with detection in the past were due primarily to the background of extraneous transcription. The level of specific transcripts is sufficient for monitoring the fractionation of extracts and identifying the factors involved in selection of different start sites in yeast as compared with HeLa extracts.

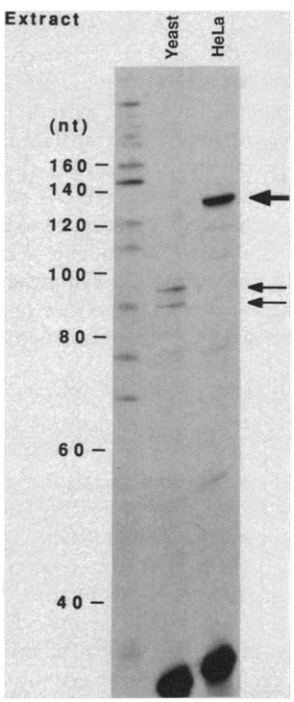

Fig. 4. Primer extension mapping of initiation sites on an adenoviral major late promoter/G-minus template in yeast and HeLa nuclear extracts. Transcripts were synthesized as in Fig. 3. Primer extension was as described (*21*), with AMV reverse transcriptase (Boehringer Mannheim), and with a 5′-end ^{32}P-labeled primer with the following sequence: 5′-GTGAGAGTGAATGATGATAGATTTGGGAAA-3′. Transcripts were analyzed by electrophoresis in a 10% polyacrylamide–7*M* urea gel in TBE buffer (*19*) and autoradiography. Bands due to G-minus transcripts in yeast and HeLa extracts are indicated by normal and bold arrows, respectively. Size markers in the leftmost lane are Msp I fragments of pBR322; nt, nucleotides.

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10. The pGC− family of plasmids contained the following series of sequence elements between the Hind III and Bam HI sites of (pUC18): Hind III–Xho I fragments bearing upstream activation sequences (UASs) as detailed below; an Xho I–Ava II fragment from pdAT-2 (*14*) containing the *CYC1* promoter, that is, residues −138 to −68 with respect to the translation start site at +1 [J. B. McNeil and M. Smith, *J. Mol. Biol.* **187**, 363 (1985)]; a synthetic oligonucleotide with the sequence GTCCTTTGTAGCATAAATTACTATACTTCTATAC, corresponding to residues −66 to −35 of the *CYC1* promoter, except with the substitution of C for G at

position −35; an Alu I–Bam HI fragment from pML(C_2AT) (*9*), consisting of 377 bp of G-minus sequence, followed by 5 bp of Sma I and Bam HI recognition sequences. The UAS fragment in pGAL4CG− was derived from pCZGAL (*15*) and contained a single GAL4-binding site. The UAS fragment in p(DED48)^{2}CG− was derived from pCZ(DED48)^2 (A. R. Buchman and R. D. Kornberg, in preparation) and contained two copies of the T-rich element from upstream of the *DED1* gene. Removal of the GAL4-binding site from pGAL4CG− by cleavage with Xba I and Eco RI, followed by filling in with the large fragment of DNA polymerase I and ligation, gave pΔCG−.

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22. We thank A. Edwards and R. Kelleher for help in developing the assay for initiation in yeast extracts with G-minus templates. We thank R. Roeder for gifts of pML(C_2AT) and p(C_2AT)19, and R. Tjian for a gift of HeLa nuclear extract. K.S. received partial support from the Toyobo Biotechnology Foundation. Costs of this research were paid from NIH grant GM36659 (R.D.K.).

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Identification of an AUUUA-Specific Messenger RNA Binding Protein

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An important control point in gene expression is at the level of messenger RNA (mRNA) stability. The mRNAs of certain regulatory cellular proteins such as oncogenes, cytokines, lymphokines, and transcriptional activators are extremely labile. These messages share a common AUUUA pentamer in their 3′ untranslated region, which confers cytoplasmic instability. A cytosolic protein was identified that binds specifically to RNA molecules containing four reiterations of the AUUUA structural element. This protein consists of three subunits and binds rapidly to AUUUA-containing RNA. Such protein-RNA complexes are resistant to the actions of denaturing and reducing agents, demonstrating very stable binding. The time course, stability, and specificity of the protein-AUUUA interaction suggests the possibility that the formation of this complex may target susceptible mRNA for rapid cytoplasmic degradation.

DESPITE GREAT PROGRESS IN ELUCIDATING the mechanisms of transcriptional regulation of gene expression, relatively little is known about post-transcriptional control at the level of mRNA turnover (*1*). In a wide variety of organisms and cell types, mRNAs display heterogeneous cytoplasmic stability (*1*). Inducible growth regulators such as oncogene products (*2*), cytokines (*3*), and transcriptional activators (*4*) tend to have extremely unstable messages with half-lives on the order of 10 to 30 min. Treatment of cells with phorbol esters (*5*), antibodies to cell surface proteins (*6*), serum (*4*), or protein synthesis inhibitors such as cycloheximide (*6, 7*) can modulate the half-lives of rapidly degraded messages. Therefore, the rates and selectivity of mRNA degradation are variable, and regulation of these processes are important control points of gene expression.

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The mechanisms by which mRNA is targeted for rapid turnover are poorly understood (*1*). A recurrent motif of rapidly degraded mRNA is an AU-rich structure in the 3′ untranslated region (*1, 8*). In particular, the pentamer AUUUA is present singly or in multiple reiterations in a wide variety of oncogene and cytokine mRNA 3′ untranslated regions. Removal of this region confers significantly greater stability to messages produced from transfected constructs (*2, 7, 8*), whereas the addition of a short DNA segment coding for this motif destabilized previously stable messages (*8*). Thus it has been proposed that a trans factor may recognize this AU-rich motif and in some way target susceptible mRNA for degradation (*1, 8*).

To determine if a cytoplasmic protein (or proteins) specifically interacts with the 3′ untranslated region of unstable mRNA through the AUUUA element, we incubated lymphocyte cytoplasmic extract with in vitro transcribed, labeled RNA that contained four adjacent reiterations of the AUUUA motif. After a brief incubation, the reaction mix was treated with ribonuclease A (RNase A) and the presence of protected complexes then assessed by band-shift assay on native, low ionic strength polyacrylamide gels (Fig. 1). A stable, RNase A–resistant complex was detected. Neither a control RNA probe of similar size, nucleotide content, and specific activity but lacking the AUUUA reiterations (Fig. 1) nor an RNA probe of similar size containing four UAAAU repeats formed stable complexes (*9*). The complex formed with the probe containing the AUUUA motifs was stable in the presence of RNase A for at least 2 hours (*9*) or when resolved in higher ionic strength running buffers such as 0.5× or 1× tris-borate-EDTA (TBE) (*9, 10*). Complex formation was abolished by prior incubation of the lysate with proteinase K (2.5 mg/ml) for 15 min (*9*).

The molecular size of the complex was assessed after ultraviolet (UV) light–induced cross-linking of the lysate-probe reaction mixture and SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2A). A stable complex migrating with a molecular mass of 28 to 45 kD was observed with the AUUUA-containing probe, but not the control probe, and stable complexes were not observed when specific or nonspecific

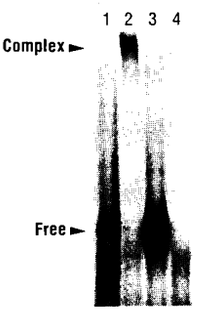

Fig. 1. Detection of protein-RNA complexes by band-shift assay. Cytoplasmic extracts of Jurkat cells were prepared by freeze-thaw lysis in 25 m*M* tris-HCl (*p*H 7.9), 0.5 m*M* EDTA, and 0.1 m*M* phenylmethylsulfonyl fluoride, followed by centrifugation at 15,000*g* at 4°C for 15 min. RNAs were transcribed by T7 RNA polymerase and labeled with [^{32}P]UTP (uridine triphosphate) to a specific activity of 10^7 cpm per microgram of RNA from Eco RI–digested pT7/T3-α19 (60-base nonspecific probe) (BRL) or Kpn I–digested pT7/T3-α19-AUUUA [64-base specific probe with four AUUUA repeats (*16*); coding oligonucleotides were cloned into the unique Sma I site]. Cytoplasmic extract (from 2×10^5 cells) was incubated with 10^4 cpm of RNA (0.5 to 1 ng), in 10% glycerol, 12 m*M* Hepes (*p*H 7.9), 15 m*M* KCl, 0.25 m*M* EDTA, 0.25 m*M* dithiothreitol, 5 m*M* $MgCl_2$, and *Escherichia coli* transfer RNA (200 ng/μl) in a total volume of 10 μl for 10 min at 30°C. RNase A was added to a final concentration of 1 μg/μl, and reaction mixtures were incubated for 30 min at 37°C before electrophoresis in a 7% native polyacrylamide gel with 0.25× TBE running buffer (*10*). Lane 1, AUUUA-containing specific probe alone; lane 2, specific probe and lysate; lane 3, nonspecific probe alone; and lane 4, nonspecific probe and lysate. The positions of complexed and free probe are indicated.