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Both a ubiquitous factor mTEF-1 and a distinct muscle-specific factor bind to the M-CAT motif of the myosin heavy chain β gene

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ABSTRACT

The A element, a fourteen base pair sequence in the rabbit myosin heavy chain (HC) β promoter (−276/−263), contains the M-CAT motif, a *cis*-acting element found in several muscle-specific genes. The A element is essential for muscle-specific transcription of the myosin HCβ gene. Recently, we have identified both muscle-specific and ubiquitous factors (A1 and A2 factors, respectively) that bind to the A element. Since the sequence of the A element is very similar to the GTIIC motif in the SV40 enhancer, we examined the relationship between A-element-binding factors and a GTIIC binding factor TEF-1, recently isolated from HeLa cells. The GTIIC motif was bound by the A1 and A2 factors in muscle nuclear extracts and competed with the A element for DNA–protein complex formation. Antibody against human TEF-1 'supershifted' the ubiquitous A2 factor–DNA complex, but did not alter the mobility of the muscle-specific A1 factor–DNA complex. We isolated a murine cDNA clone (mTEF-1) from a cardiac cDNA library. The clone is highly homologous to Hela cell TEF-1. The *in vitro* transcription/translation product of mTEF-1 cDNA bound to the A element, and the DNA binding property of mTEF-1 was identical to that of the A2 factor. Transfection of mTEF-1 cDNA into muscle and non-muscle cells confirmed that mTEF-1 corresponds to A2, but not to A1 factors. The mTEF-1 mRNA is expressed abundantly in skeletal and cardiac muscles, kidney and lung, but it is also expressed at lower levels in other tissues. These results suggest that the M-CAT binding factors consist of two different factors; the ubiquitous A2 is encoded by mTEF-1, but the muscle-specific A1 factor is distinct from mTEF-1.

INTRODUCTION

Significant progress has been made in delineating the regulators of skeletal muscle commitment and differentiation with the identification of a family of muscle specific E-box binding proteins, the MyoD family (MyoD, myogenin, myf-5, and MRF4) (1–5). However, not all muscle genes have an E-box in the regions known to be critical for their tissue-specific expression (6, 7). In addition, no members of this family are expressed in cardiac muscle cells (8), although cardiac and skeletal muscles express many muscle-specific genes in common during development. Thus, it is likely that factors other than MyoD family are also involved in regulation of muscle specific genes.

Other regulatory factors, such as MEF-2/RSRF (9, 10) which binds to an AT-rich sequence motif found in several muscle-specific promoters (11), appear to be induced by members of the MyoD family and positively regulate expression of muscle-specific genes (10, 12). There is evidence that ubiquitous factors are also essential for skeletal muscle specific expression (13, 14). One such factor, serum response factor (15), has been shown to bind to the CArG box/CBAR and is required for muscle-specific expression of the skeletal and cardiac actin promoter (16–18). There is also evidence for the presence of the regulatory factors that are involved in muscle-specific gene expression, but are independent of the E-box regulatory pathway. One such factor is the M-CAT binding factor, which binds to the M-CAT (muscle CAT) motif CATTCCT originally identified in the chicken cardiac troponin-T (cTnT) promoter (19).

Myosin HCβ is a major contractile protein in cardiac and slow skeletal muscle. In some cases, mutations in the myosin HCβ gene have been shown to be the cause of familial hypertrophic cardiomyopathy (20, 21). It has been shown that expression of the myosin HCβ gene is controlled by multiple *cis*-acting regulatory elements in the 5′ flanking region (6, 22–24). In the myosin HCβ gene of rat, rabbit and human, the region within 295 bp upstream from the transcriptional initiation site of the gene is sufficient to direct both tissue- and developmental stage-specific expression of this gene in cultured skeletal and cardiac muscles (6, 22–24). We have recently shown that at least five positive *cis*-acting elements are required for the transcription of the rabbit myosin HCβ gene (6). Two of these elements, referred to as A (−276/−263) and B (−207/−180), are essential for conferring muscle-specific activation on homologous and heterologous

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promoters (6). Since these elements have no homology to the E box, it is likely that the muscle-specific expression of myosin HCβ gene is not directly dependent on the MyoD family (6, 24).

We have also identified nuclear protein factors that interact with the A and B elements by gel mobility shift and methylation interference analyses. By using the A element as a probe and nuclear extracts from muscle cells, we found two protein–DNA complexes in a gel mobility shift assay that had identical methylation interference patterns (25). We referred to the factors which are responsible for the forming these two complexes as A1 and A2 factors. The A1 factor is found mainly in nuclear extracts from differentiated muscle cells. In contrast, the A2 factor is observed in nuclear extracts from undifferentiated muscle and non-muscle cells. Thus, both muscle-specific A1 factor and ubiquitous A2 factor bind to the same DNA binding site (25).

The A element contains a sequence similar to the M-CAT motif (see Fig 1A), which is essential for the muscle-specific expression of cTnT and myosin HCβ genes in cardiac and skeletal muscle (19, 24, 25). The M-CAT motif in the A element is also similar to the GTIIC motif (see Fig 1A) in the SV40 enhancer (23, 24). We have also identified a B-element-binding factor that is expressed ubiquitously (25). Both A and B elements are essential for muscle-specific expression of myosin HCβ gene (6). Therefore, interaction between muscle-specific A1 factor and the ubiquitous A2 and B factors may be involved in the muscle-specific transcription of this gene (25).

Xiao *et al.* (26) have cloned the cDNA encoding the human transcriptional enhancer factor 1 (TEF-1), which binds to the GTIIC motif and the Sph enhanson in the SV40 enhancer. Since the core sequence of A element is very similar to the GTIIC motif, we examined whether A1 and A2 factors are related to human TEF-1. While this work was in progress, Farrance *et al.* (27) reported that the chicken M-CAT binding factor is related to TEF-1 by immunological analysis, DNA binding specificity and DNA-agarose chromatography. They suggested that TEF-1 may be the sole component of the M-CAT binding activity. However, our observations indicate that there are two distinct M-CAT binding factors (A1 and A2). To address the question of whether one or both of the factors we had identified is TEF-1 itself or a related protein, we isolated and characterized TEF-1 related cDNAs (mTEF-1) from a mouse cardiac muscle cDNA library and examined the relationship between mTEF-1 and the A-element-binding factors (A1 and A2). Our results demonstrate that mTEF-1 encodes the ubiquitous A2 factor. However, the muscle-specific A1 factor is different from mTEF-1. Thus, the M-CAT binding factors consist of both a ubiquitous factor mTEF-1 and a muscle-specific factor A1.

MATERIALS AND METHODS

Cell culture

Mouse skeletal muscle (Sol8 and C2C12), mouse fibroblast (BALB/c 3T3) and human cervical carcinoma (HeLa) cell lines were maintained in growth medium containing Dulbecco modified Eagle medium (GIBCO Laboratories) with either 20% (Sol8 and C2C12 cells) or 10% (3T3 and HeLa cells) fetal bovine serum. Myogenic differentiation of Sol8 and C2C12 myoblasts was induced by exposure of confluent cultures to differentiation medium containing Dulbecco modified Eagle medium and 10% horse serum.

Preparation of nuclear extracts and heparin-agarose chromatography

The crude nuclear extracts from Sol8 and HeLa cells were prepared essentially as described by Dignam *et al.* (28). The fractionation of nuclear extracts on a heparin-agarose column were performed as previously described (25).

Gel mobility shift assay

Binding reactions were carried out as previously described (25). Gels were run at 150V in a 0.5×TBE buffer (29). For 'supershift' experiments, fractionated nuclear extracts were preincubated with either anti-TEF-1 or nonimmune serum at room temperature for 30 min before addition of labeled DNA probe and poly(dI-dC).

The sequences of the oligos used for gel mobility shift assays are as follows:

A	5′	gatcCAGGCAGTGGAATGCGAGGAG	3′
	3′	GTCCGTCACCTTACGCTCCTCctag	5′
MutA	5′	gatcCAGGCAGTTCAACGCGAGGAG	3′
	3′	GTCCGTCAAGTTGCGCTCCTCctag	5′
GTIIC	5′	CCAGCTGTGGAATGTGTGT	3′
	3′	GGTCGACACCTTACACACA	5′
B	5′	agctCCTGCACACCCCATG	3′
	3′	GGACGTGTGGGGTACtcga	5′

The nucleotides shown in lowercase letters are not present in the gene. The altered nucleotides are underlined.

Western blot analysis

One hundred and fifty micrograms of partially purified nuclear extracts from HeLa cells and Sol8 myotubes were electrophoresed on an 11% sodium dodecyl sulfate (SDS)-polyacrylamide gel (30) and electroblotted onto nitrocellulose as described by Towbin *et al.* (31). The blots were blocked with 3% nonfat milk in PBS for 1 h at room temperature, and then incubated at 37°C with TEF-1 antisera (anti-P2), kindly provided by Dr Chambon (26). After washing with 0.05% Tween-20 in PBS at room temperature, the blots were incubated with ^{125}I-labeled protein A for 1 h and washed again.

Southwestern blot analysis

Southwestern blot analysis was performed as described by Silva *et al.* (32). Fifty micrograms of crude nuclear extracts were separated on a 7.5% SDS-polyacrylamide gel, treated with a renaturation solution (32) , and transferred to the nitrocellulose filter as described by Towbin *et al.* (31). The blots were blocked with a binding buffer containing 50 mM NaCl, 10 mM Tris–HCl, pH 7.4, 1 mM EDTA and 5% nonfat milk for 2 h, then incubated with end-labeled oligo A or MutA in the binding buffer for 3 h at room temperature.

Cloning of mouse TEF-1

The 5′ 671 bp EcoRI-BamHI fragment of human TEF-1 cDNA, kindly provided by Dr P.Chambon (26), was labeled by the random oligo priming method and used to screen an adult mouse cardiac λZAPII cDNA library (Stratagene). Prehybridization was performed at 32°C in a solution containing 50% formamide, 0.2% polyvinyl-pyrrolidone, 0.2% bovine serum albumin, 0.2% Ficoll, 0.05 M Tris–HCl, pH 7.5, 1.0 M NaCl, 0.1% sodium pyrophosphate, 1.0% SDS, 10% dextran sulfate and 100 μg/ml of denatured salmon sperm DNA. Hybridization was performed at 32°C in the same solution with denatured probe. The filter

was washed in 2×SSC (1×SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS at room temperature, followed by washing in either the same solution at 50°C (low stringency condition).

DNA sequencing

The nested deletions of plasmid mTEF1-11 were created using ExoIII/mung bean nuclease deletion kit (Stratagene) and double-stranded DNA sequencing was performed using the Sequenase kit (United States Biochemical Corp.).

Northern blot analysis

Total RNA from cells was extracted according to Chirgwin *et al.* (33). Total RNA from different tissues of adult mouse was extracted according to Chomczynski and Sacchi (34). Thirty micrograms of total RNA was electrophoresed on 0.8% or 1% agarose-1.1M formaldehyde gels, and transferred to nylon filters. The mTEF-1 probe was prepared by randam oligo priming method. Prehybridization and hybridization were performed at 42°C in the same solution as described for cDNA cloning (see above). The filters were washed in 2×SSC and 0.1% SDS at room temperature, followed by washing in 0.1×SSC and 0.1% SDS at 60°C.

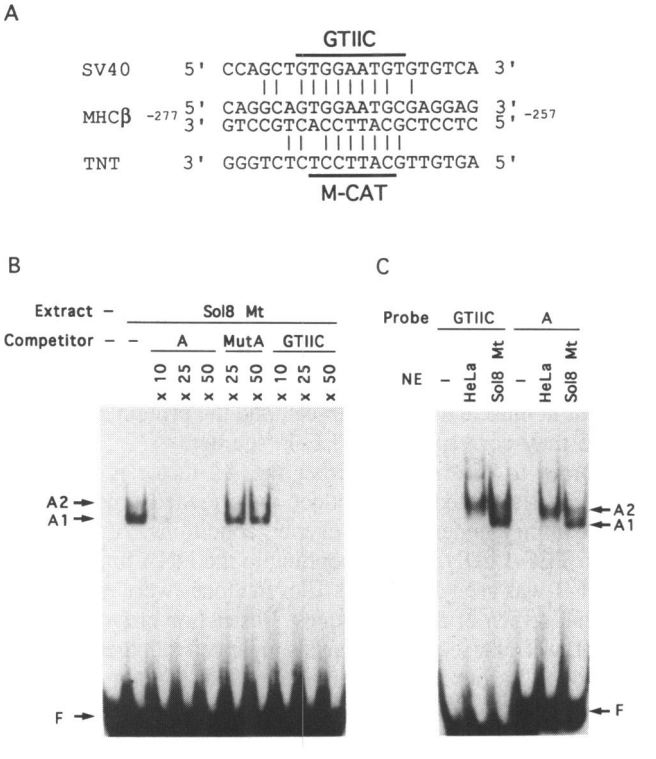

Figure 1. A element of myosin HCβ gene and A-element-binding factors. **(A)** Comparison of A element in myosin HCβ gene (MHCβ) with the GTIIC motif in the SV40 enhancer and the M-CAT motif in cardiac troponin T (TNT) promoter. The lines indicate sequences similar to GTIIC and M-CAT (in reverse orientation) motifs. **(B)** Competition by various oligos for complex formation between nuclear proteins and the A element. End-labeled oligo A was incubated with 2.5 μg of nuclear extracts from Sol8 myotubes (Mt) in the absence (–) or presence of unlabeled competitors (oligo A, MutA, and GTIIC) at a 10-, 25- or 50-fold molar excess over the labeled probe. Arrows indicate the positions of complexes (A1 and A2) and free probe (F). **(C)** Gel mobility shift analysis of nuclear extracts from HeLa and Sol8 myotube with A element and GTIIC. End-labeled oligo GTIIC or oligo A was incubated with 2.5 μg of nuclear extracts from HeLa and Sol8 myotubes and analyzed on an 8% native polyacrylamide gel. Note HeLa cell extracts yield only the A2 complex, while Sol8 myotube extracts yield both A1 and A2 complexes.

In vitro transcription and translation

Plasmid mTEF-11 was linearized with NdeI, downstream of the stop codon, and transcribed using T7 RNA polymerase in the presence of m7G(5′)ppp(5′)G in addition to the NTPs. One microgram of RNA product was translated with 20 μl of rabbit reticulocyte lysate (Stratagene) in the presence of [^{35}S] methionine.

Construction of plasmids

The mTEF-1 expression plasmid pCMV-TEF1 was constructed by insertion of the XhoI–XbaI fragment of plasmid mTEF1-11 into the XhoI–XbaI site of the eukaryotic expression vector pcDNAI (Invitrogen Corp.) driven by the human cytomegalovirus (CMV) promoter.

Transfection and enzyme assay

DNA transfection was performed by the calcium phosphate precipitation method (35) as previously described (25). For overexpression of mTEF-1 in cultured cells, 30 μg of plasmid per 100-mm dish was transfected into either HeLa cells or Sol8 myoblasts. For co-transfection experiment, different amount of the mTEF-1 expression plasmid (0.1, 0.5, 1.0 and 2.0 μg), a reporter CAT plasmid (2.5 or 5.0 μg) and an internal control plasmid, pCMV-lacZ (1 μg) were transfected in 60-mm dish. The activity of chloramphenicol acetyltransferase (CAT) in the cell extracts was assayed as described by Gorman *et al.* (36). The activity of b-galactosidase in the cell extracts was assayed according to the method of Miller (37).

Nucleotide sequence accession number

The GenBank accession number for the sequence presented in this report is L13853.

RESULTS

The A element-binding factors interact with the GTIIC motif in the SV40 enhancer

The A element found in the myosin HCβ promoter is identical to the GTIIC motif, 5′ GTGGAATGT 3′ located immediately upstream of the 72 bp repeat of the SV40 enhancer, except for one base mismatch (underlined) (Fig.1A). To investigate the relationship between A element and GTIIC binding proteins, we performed DNA binding competition experiments between labeled oligo A and unlabeled oligos A, MutA, or GTIIC in gel mobility shift assays (Fig. 1B). As previously indicated (25), when oligo A probe was incubated with nuclear extracts from Sol8 myotubes, two distinct protein–DNA complexes (A1 and A2) were observed (Fig. 1B, lane 2). Oligo GTIIC competed with labeled oligo A for formation of A1 and A2 complexes as efficiently as did unlabeled oligo A, whereas oligo MutA had no effect on the formation of complexes. The A1 complex was only seen in differentiated muscle cells (Sol8 myotubes), whereas the A2 complex was seen in both muscle (Sol8 myotubes) and non-muscle (HeLa) cells (Fig. 1C right, and see also ref 25). In addition, when oligo GTIIC was used as a probe, two

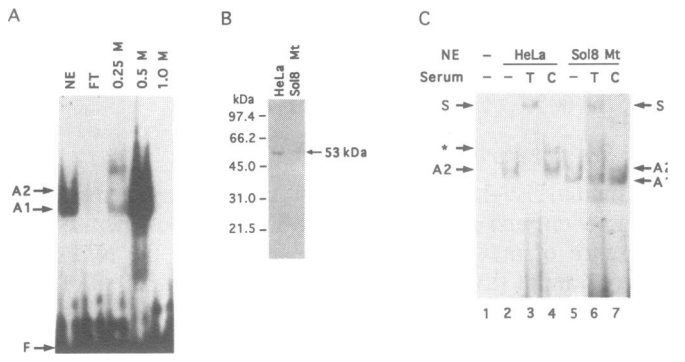

Figure 2. Relationship between TEF-1 and A-element-binding factors. **(A)** Heparin-agarose column profile of DNA binding activities. Nuclear extracts from Sol8 myotubes (NE) were applied to a heparin-agarose column and proteins bound to the column were eluted in a stepwise manner with 0.25 M, 0.5 M, and 1.0 M NaCl. Three micrograms of each fraction were tested for the ability to bind to oligoA by a gel mobility shift assay. FT, flow through. Arrows indicate the position of complexes (A1 and A2) and free probe (F). **(B)** Western blot analysis of TEF-1. Partially purified nuclear extracts from HeLa cells or Sol8 myotubes (Mt) were separated on an 11% SDS-polyacrylamide gel and transferred to nitrocellulose. The filter was incubated with anti-TEF-1 antibody followed by ^{125}I-labeled protein A. **(C)** Effect of the anti-TEF-1 serum on the binding of nuclear factors to the A element. Oligo A was end-labeled and used as a probe. Two micrograms of partially purified nuclear extracts (NE) from either HeLa cells or Sol8 myotubes (Mt) were preincubated with anti-TEF-1 serum (T) or nonimmune control serum (C), then incubated with a probe and analyzed on an 8% native polyacrylamide gel. Arrows indicate the positions of specific complexes (A1 and A2), supershifted (S), and non-specific (*) complexes. Note supershifted bands (arrow S) are seen only in the presence of TEF-1 antisera (T).

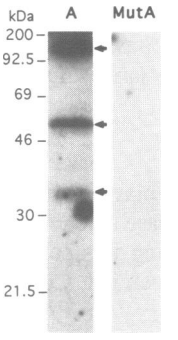

Figure 3. Southwestern blot analysis of crude nuclear extracts from Sol8 myotubes. The crude nuclear extracts from Sol8 myotubes were separated by SDS-polyacrylamide gel, transferred to a nitrocellulose filter, and hybridized with end-labeled oligoA or MutA (mutated version of A). Arrows indicate the locations of protein bands hybridizing to the probes.

DNA−protein complexes were formed (Fig 1C, lane 3). Their mobilities, cell type specificity, and competition profile were very similar to those obtained by using oligo A as a probe (Fig. 1C and data not shown). These results suggest that the GTIIC motif binds to the A-element-binding factors.

The A2 Factor is related to TEF-1

Because the transcriptional enhancer factor 1 (TEF-1) isolated from HeLa cells binds to the GTIIC motif (26), we examined whether or not the A factors are related to TEF-1 by using an antibody against human TEF-1. First, the reactivity of the antibody against human TEF-1 was examined by Western blot analysis. Nuclear extracts from HeLa cells and Sol8 myotubes were partially purified by heparin-agarose column chromatography as described previously (25). The A1 and A2 factors bound to the A element were found mainly in the 0.5 M NaCl fraction (Fig.2A). The 0.5 M NaCl fraction was separated on an 11% SDS-polyacrylamide gel and transferred to nitrocellulose. The filter was incubated with anti-TEF-1 antibody followed by ^{125}I-labeled protein A. In both HeLa cells and Sol8 myotubes, a ~53 kDa protein, the expected size of TEF-1 (26), reacted with the anti-TEF-1 serum (Fig. 2B). Therefore, this antibody recognizes both human and mouse TEF-1. In fact, the peptide sequence of human TEF-1 used for raising the antiserum (a.a. residues 16−25) (26) was identical to that of mouse TEF-1 (see below). The mouse TEF-1 appears slightly larger than human TEF-1 (Fig 2B.), probably because the former is 4 amino acids larger than the latter (see below).

Using this anti-TEF-1 antibody, we then examined whether the A factor−DNA complexes could be 'supershifted' in a gel mobility shift assay. As shown in Fig. 2C, the A2 factor complex in nuclear extracts from HeLa cells and Sol8 myotubes was supershifted (marked by arrow S) by the anti-TEF-1 antibody (lanes 3 and 6), but not by nonimmune control serum (lanes 4 and 7). In contrast, the muscle-specific A1 factor complex in nuclear extracts from Sol8 myotubes was not supershifted by anti-TEF-1 (lane 6). These results suggest that the ubiquitous A2 factor is antigenically related to TEF-1 but muscle-specific A1 factor seems distinct from TEF-1.

In order to estimate the number and molecular mass of proteins that bind to the A element, we analyzed the nuclear proteins from Sol8 myotubes by Southwestern blot analysis. End-labeled oligo A interacted with three protein bands of approximately 32, 53 and 120 kDa (Fig. 3, left). In contrast, oligo MutA (mutated version of oligo A) did not detect these proteins (Fig. 3, right). Thus, there seem to be multiple proteins that bind to the A element in muscle nuclear extracts, and the protein of ~53 kDa in size may correspond to TEF-1 (see below).

In order to determine whether the A2 factor is identical to TEF-1 or is a related gene product, we screened a mouse cardiac cDNA library under low stringency conditions. A fragment of human TEF-1 cDNA corresponding to the DNA binding region of TEF-1 was used as a probe. Eleven clones were isolated from an initial screen of 5×10^5 plaques. Restriction enzyme mapping and partial DNA sequence analysis indicated that these 11 clones correspond to four independent clones that contain overlapping cDNA fragments derived from a common transcript (data not shown). Clone mTEF-11, contained the largest insert (2.8 kb). Nested deletions were made of this clone and the DNA sequence of the insert was determined (Fig. 4).

The nucleotide sequence of the mTEF-11 cDNA contains an open reading frame of 1290 bp encoding a 430 amino acid protein. The deduced amino acid sequence of TEF-1 shows a striking homology (98.4% identity) between the mouse and human proteins. Mouse TEF-1 differs from human TEF-1 by 7 amino acid residues. Four of these are contiguous and appear to be deleted in the human sequence (Fig. 4, dotted a.a.). The nucleotide sequence corresponding to the amino-terminal region of TEF-1, as well as the immediately upstream sequence of the putative initiation codon (nucleotides 134−188 in clone mTEF-11) are conserved between mouse and human TEF-1. Therefore, we consider it likely that AUU (Ile) at 189, rather than AUG (Met) at 234, is used as an initiation codon, as has

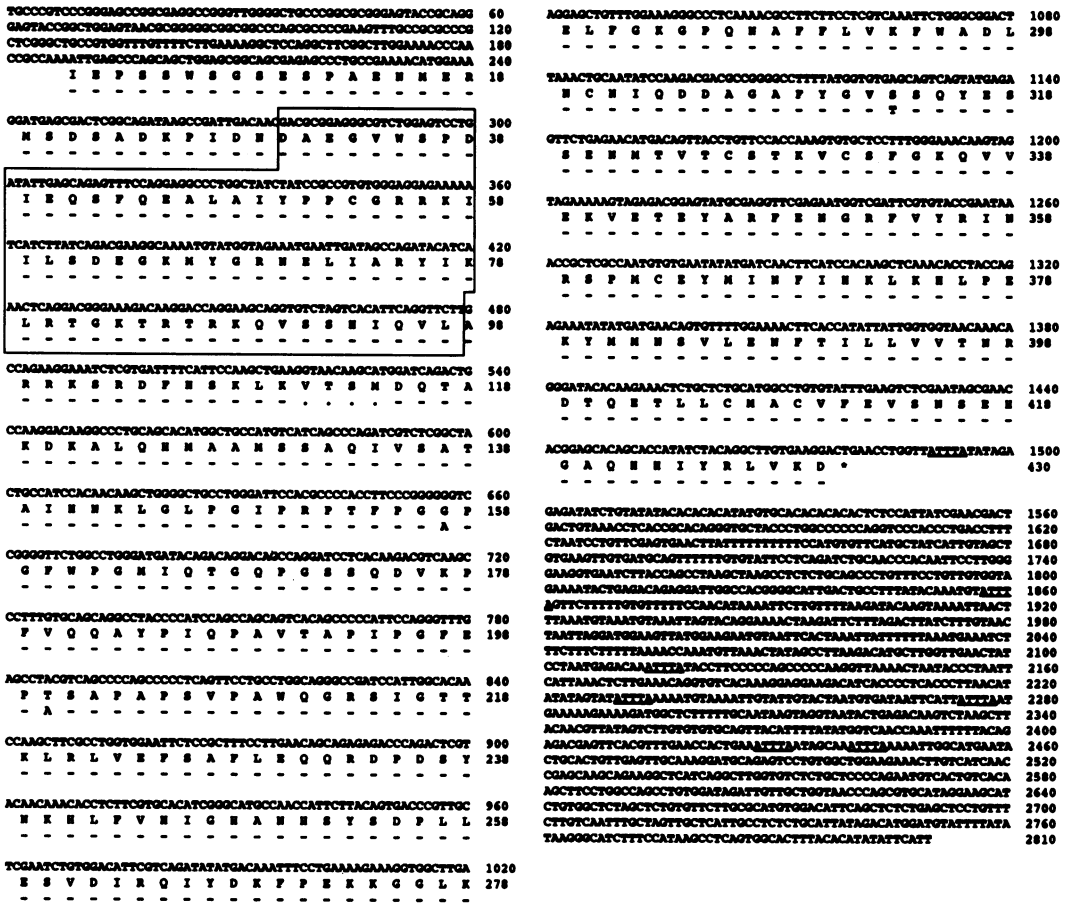

Figure 4. Nucleotide and predicted amino acid sequence of mTEF-1. The top and middle lines indicate nucleotide and amino acid sequence of mTEF-1, respectively. The bottom line shows amino acid sequence of human TEF-1. Dashes, identical sequences between mouse and human (26); Dots, the absence of amino acids; asterisk, the stop codon for translation. The TEA domain is boxed. Potential mRNA destabilization signals (AUUUA) in the 3′ untranslated region are underlined. While this manuscript was in revision, we became aware that the amino acid sequence of mTEF-1 cloned from a mouse cDNA library of PCC4 embryonal carcinoma cells was reported (46). Our mTEF-1 sequence differs from that of PCC4 in four contiguous amino acids (a.a.111−114 in our sequence), which are absent in mTEF-1 from PCC4.

been demonstrated for the human protein (26). The TEA domain, which seems to be an evolutionarily conserved DNA binding motif, (38) is present between residues 30 and 97 (Fig. 4).

mTEF-1 transcript is expressed in both muscle and non-muscle cells

We examined the expression of the mouse TEF-1 (mTEF-1) transcripts in various tissues and cell lines by Northern blot analysis using a probe containing 5′ untranslated and coding regions from the mTEF-11 clone. A ~12 kb transcript was observed in all tissues and cell lines tested. This transcript is abundant in both skeletal and heart muscles, as well as in kidney and lung. It is also detectable at low levels in other organs (Fig. 5A). In the case of cell lines, mTEF-1 transcript is expressed in both the myoblasts and myotubes of skeletal muscle cells, (C2C12 and Sol8) as well as in non-muscle cells (3T3 and HeLa) (Fig. 5B).

Both mTEF-1 and A2 factor form similar complexes with the A element

We examined whether *in vitro* translated mTEF-1 binds to the A element by gel mobility shift assay. When *in vitro* transcription/translation products of mTEF-11 cDNA were analyzed by an SDS-polyacrylamide gel, a major ~53 kDa band and minor ~51 kDa and ~40 kDa bands were observed (Fig. 6A). The ~51 and ~40 kDa bands may correspond to the translation products that are initiated from internal methionine residues. When these *in vitro* translated products were incubated with an A element probe, they formed the protein−DNA complex with the same mobility as that of the A2 factor (Fig. 6B, lane 3).

In order to examine whether A1 factor is a product of a post-translational modification of mTEF-1 *in vivo*, we overexpressed the cloned mTEF-1 cDNA in cultured cells. HeLa cells and Sol8 myocytes were transiently transfected with an eukaryotic expression vector with or without a mTEF-1 cDNA insert (pCMV-mTEF1 or pcDNAI, respectively). Nuclear extracts were prepared from these transfected cells and subsequently analyzed by gel mobility shift assay. In nuclear extract from Sol8 myotubes transfected with the mTEF-1 expression vector, the amount of A2 complex increased significantly, while the amount of A1 complex remained constant (Fig. 7, left). Similarly, the amount of A2 complex increased in nuclear extract from HeLa cells transfected with the mTEF-1 expression vector, while the amount of B factor, which binds to the B element in myosin HCβ promoter (25), remained unchanged (Fig. 7, right panel). Thus,

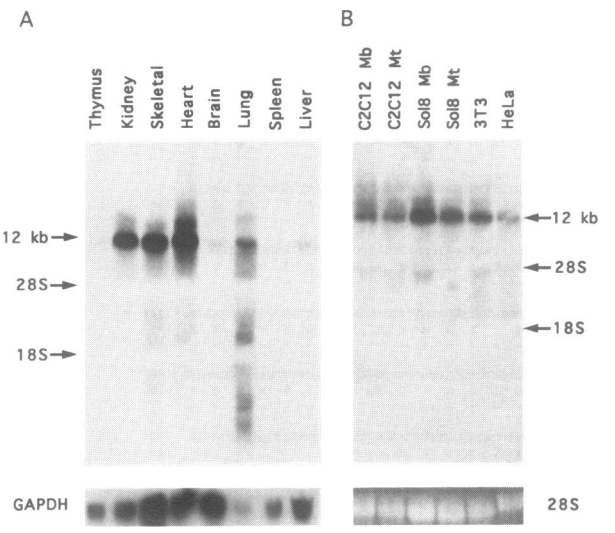

Figure 5. Northern blot analysis of mTEF-1 transcripts. Thirty micrograms of total RNAs extracted from different mouse tissues (**A**) and cell lines (**B**) were separated on either 0.8% or 1% agarose-formaldehyde gel. A 858 bp EcoRI fragment from plasmid mTEF1-11 was used as a probe. The positions of both 28S and 18S ribosomal RNAs are indicated. The mTEF-1 probe hybridizes to 12kb mRNA species in all tissues. Multiple lower bands seen in Lung lane are probably due to partial degradation of lung RNA. In panel A, the control hybridization of the same filter using the GAPDH cDNA as a probe is shown at the bottom. In panel B, the ethidium bromide staining of 28S ribosomal RNA of the same gel is shown at the bottom.

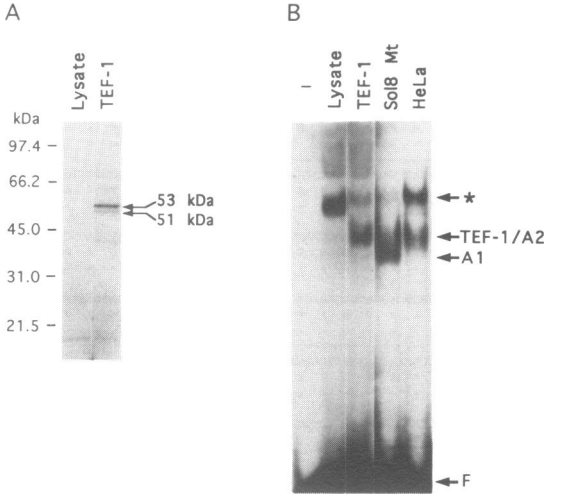

Figure 6. Transcription/translation of mTEF-1 *in vitro*. (**A**) SDS-polyacrylamide gel of *in vitro* translated mTEF-1. The mTEF-1 RNA synthesized *in vitro* using T7 RNA polymerase was translated in rabbit reticulocyte lysate and 3 μl of the *in vitro* translation products was analyzed on an 11% SDS-polyacrylamide gel. (**B**) Gel mobility shift assay of *in vitro* translated mTEF-1 with oligo A. Three microliters of either unprogramed rabbit reticulocyte lysate (Lysate), mTEF-1 translation products (TEF-1) or 2 μg of nuclear extracts from HeLa and Sol8 myotubes (Mt) were incubated with end-labeled oligo A and analyzed on an 8% native polyacrylamide gel. Arrows indicate the position of the complexes (A1 and A2), non-specific complex (*) and free probe (F).

mTEF-1 expressed *in vivo* is indistinguishable from the A2 factor and it is unlikely that the A1 factor is a product of post-translational modification of mTEF-1 *in vivo*.

Human TEF-1 has been shown to activate transcription of the SV40 promoter (26) and human papilloma virus-16 *E6* and *E7* oncogene promoters (43) in collaboration with a limiting cell specific co-activator. Transfection of increasing amounts of human TEF-1 resulted in transcriptional repression of these promoters presumably by 'squelching' the putative coactivator (26, 43). We therefore examined whether mTEF-1 affects transcription of myosin HCβ promoter by a transient transfection assay. The mTEF-1 expression vector (pCMV-mTEF1) and the CAT reporter plasmids were co-transfected into either Sol8 myoblasts or HeLa cells. Unexpectedly, the co-transfection of increasing amounts of pCMV-mTEF-1 (0.1, 0.5, 1.0 and 2.0 μg/dish) had no effects on the expression of MHCβ-CAT construct driven by myosin HCβ promoter (−628) in Sol8 myotubes (Fig. 8A). Activity of myosin HCβ promoter was very low in HeLa cells (6, 25) and it was not significantly transactivated by co-transfection of pCMV-mTEF-1 (Fig. 8B). In contrast, the expression of GTIIC-TKCAT plasmid containing 5 copies of GTIIC tandem repeats upstream of thymidine kinase (TK) promoter (26) was repressed efficiently ('squelching' effects) by the co-transfection of pCMV-mTEF-1 in both Sol8 myotubes and HeLa cells (Fig. 8A and B). Unlike the pGTIIC-TKCAT, 3A-TKCAT construct containing 3 copies of A element (M-CAT motif) upstream of TK promoter was preferentially expressed in Sol8 myotubes than in HeLa cells (20-fold and 4.6-fold increase over the parental plasmid TKCAT, respectively). Co-transfection of increasing amounts of mTEF-1 expression vector slightly reduced the expression of p3A-TKCAT. However, the effect was not as prominent as that of the pGTIIC-TKCAT in both Sol8 and HeLa cells (Fig. 8A and B).

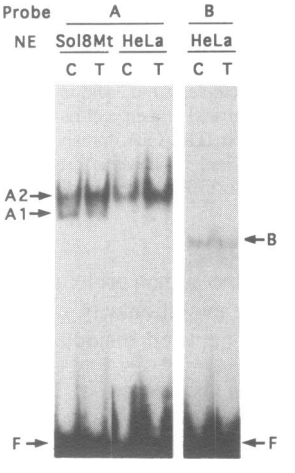

Figure 7. Overexpression of the mTEF-1 *in vivo*. Nuclear extracts were prepared from HeLa cells and Sol8 myotubes transfected with either expression vector containing mTEF-1 cDNA (pCMV-TEF1) (T) or the expression vector alone (pcDNAI) (C). The nuclear extracts were incubated with labeled oligo A or B and analyzed on an 8% native polyacrylamide gel. Arrows indicate the position of the complexes (A1, A2, B) and free probe (F). Note selective increases of the A2 complex in the extracts from transfected cells (T).

DISCUSSION

To investigate the relationship between A element-binding proteins (A1 and A2 factors) and an SV40 enhancer binding protein TEF-1, we have screened a mouse cardiac cDNA library and isolated a cDNA containing the complete coding region of mouse TEF-1. A single open reading frame of 1290 nucleotides

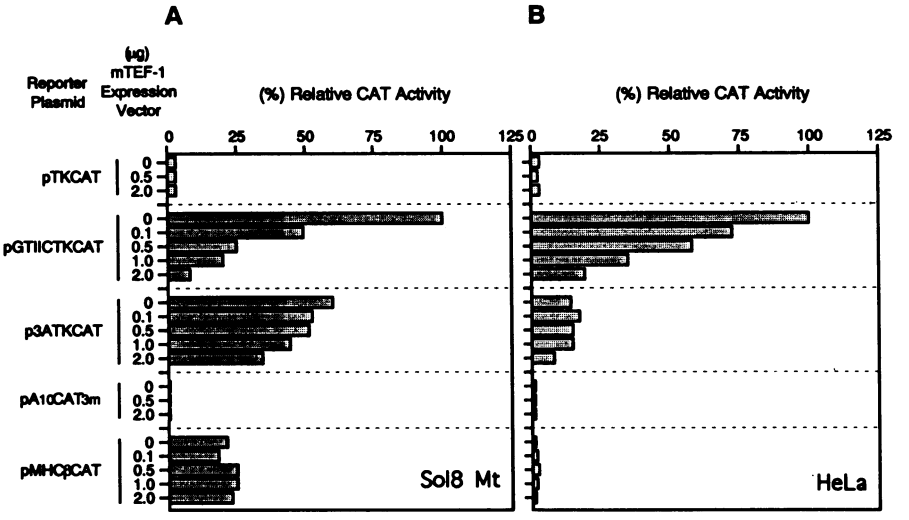

Figure 8. Effect of the mTEF-1 on the expression of myosin HCβ promoter. Each reporter plasmid was transfected with different amount of mTEF-1 expression vector and pCMV-lacZ into either Sol 8 myocytes **(A)** or HeLa cells **(B)**. The pGTIIC-TKCAT and p3A-TKCAT contain 5 copies of GTIIC tandem repeats and 3 copies of A element (21bp), respectively, upstream of TK promoter in pTKCAT. The pA10CAT3m is a parental plasmid for pMHCβCAT. The CAT activity was normalized by the β-galactosidase activity for each transfection. All CAT activities are given relative to the values obtained for the pGTIICTKCAT, which is set at 100% for each cell types. Expression values represent the averages of 2−4 separate transfection experiments. Variation was less than 10% between experiments.

encodes a protein of 430 amino acids, 4 amino acids longer than human TEF-1. The primary structure of TEF-1 is highly conserved between mouse and human. The amino terminal portion of TEF-1 contains a TEA DNA-binding domain (38, 39). This domain has been found in three other proteins, all of which appear to be transcription factors: ABAA, involved in spore differentiation in *Aspergillus* (40); TEC1, required for Ty1 enhancer activity in yeast (40); and *scalloped* protein, involved in neural development in *Drosophila* (42).

We conclude that A2 factor is encoded by mTEF-1 for several reasons. First, both the A2 factor and TEF-1 bind to the GTIIC motif (Fig 1B, C). Second, an antibody specific for TEF-1 quantitatively supershifts the DNA−A2 factor complex (Fig 2C, lane 3). Third, the *in vitro* transcription/translation product of mTEF-1 cDNA clone interacts with the A element, and the mobility of its protein−DNA complex is identical to that of A2 factor (Fig 6). Fourth, the expression pattern of mTEF-1 mRNA is consistent with that of A2 factor activity in various cell lines (Fig 5, ref 25 and data not shown). Finally, overexpression of mTEF-1 *in vivo* selectively increases the amount of A2 factor−DNA complex in gel mobility shift assays (Fig 7).

Recently, we have reported that A1 factor and A2 factor share identical DNA binding properties in oligonucleotide competition and methylation interference patterns (25). Our results suggest that the A1 factor is distinct from mTEF-1 because the antibody specific for TEF-1 did not supershift the A1-factor−DNA complex. In addition, the A1 factor is present only in differentiated muscle cells, whereas mTEF-1 is expressed in a variety of cell types. Moreover, overexpression of mTEF-1 in Sol8 myotubes did not increase the A1 factor complex but increased the A2 factor complex in gel mobility shift assays.

Recently, Farrance *et al.* (27) have reported that the M-CAT binding factor is immunologically and biochemically related to TEF-1. Our results presented here provide definitive evidence that TEF-1 is a M-CAT binding factor. However, our results differ from those of Farrance *et al.* in one important point. While Farrance *et al.* suggest that TEF-1 may be the only factor binding to the M-CAT motif (27), our results clearly demonstrate that the M-CAT factor consists of two distinct factors, the ubiquitous factor mTEF-1 and the muscle-specific A1 factor. This may account for the apparent lack of transactivation of myosin HCβ promoter by overexpression of mTEF-1 alone, because the M-CAT element is likely to require both mTEF-1 and the muscle-specific A1 factor to activate muscle-specific genes. Our results also suggest a potentially different function of TEF-1 on a muscle-specific promoter (the lack of 'squelching' effect on pMHCβ-CAT, Fig.8) and on the viral promoters (Fig. 8 and 26, 43).

Though A1 factor and mTEF-1 are distinct, it is possible that they may still be related. One possibility is that A1 factor is a different gene product from mTEF-1, but is still a member of the family of transcription factors with the TEA DNA-binding domain. This hypothesis is based on our observations that (i) the methylation interference pattern was the same between A1 and A2/TEF-1 factors (25), (ii) Southwestern blot analysis showed two additional A-element-binding factors (~120 and ~32 kDa proteins) (Fig 3) and (iii) genomic Southern blot analysis showed the presence of other mTEF-1 related genes (data not shown). Very recently, we have isolated a TEA domain containing cDNA distinct from mTEF-1 (our unpublished observation). It remains to be determined whether this clone corresponds to the A1 factor. The second possibility is that the A1 factor and mTEF-1 represent alternatively spliced products of the same gene. In this case, the epitope(s) recognized by the anti-TEF-1 antibody used in this study must reside in a differentially spliced exon. However, we did not isolate such a clone using the cardiac muscle cDNA library.

A requirement for a tissue-specific intermediary factor(s) has been reported for transactivation by TEF-1 (26, 43). It is known that heterodimerization between ubiquitous and tissue-specific factors with similar structural motifs can regulate tissue-specific transcription. Such dimerization has been reported for the transcription factors with the helix-loop-helix motif (MyoD and

E12) (13, 44) and Pou domain (Pit-1 and Oct-1) (45). It is possible that mTEF-1 might interact with a muscle-specific, TEA domain containing factor to form a functional heterodimer to bind the M-CAT motif of muscle-specific genes.

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REFERENCES

1. Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E., and Arnold, H.H. (1989) *EMBO J.* **8**, 701–709.
2. Davis, R.L., Weintraub, H., and Lassar, A.B. (1987) *Cell* **51**, 987–1000.
3. Rhodes, S.J. and Konieczny, S.F. (1989) *Genes Dev.* **3**, 2050–2061.
4. Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezta, R., Blackwell, T.K., Turner, D., Rupp, R., Hollenberg, S., Zhuang, Y. and Lassar, A. (1991) *Science* **251**, 761–766.
5. Wright, W.E., Sassoon, D.A. and Lin, V.K. (1989) *Cell* **56**, 607–617.
6. Shimizu, N., Prior, G., Umeda, P.K. and Zak, R. (1992) *Nucleic Acids Res.* **20**, 1793–1799.
7. Uetsuki, T., Nabeshima, Y., Fujisawa-Sehara, A. and Nabeshima, Y. (1990) *Mol. Cell. Biol.* **10**, 2562–2569.
8. Sassoon, D., Lyons, G., Wright, W.E., Lin, V., Lassar, A., Weintraub, H. and Buckingham, M. (1989) *Nature (London)* **341**, 303–307.
9. Pollock, R. and Treisman, R. (1991) *Genes Dev.* **5**, 2327–2341.
10. Yu, Y.-T., Breitbart, R.E., Smoot, L.B., Lee, Y., Mahdavi, V. and Nadal-Ginard, B. (1992) *Genes Dev.* **6**, 1783–1798.
11. Gossett, L.A., Kelvin, D.J., Sternberg, E.A. and Olson, E.N. (1989) *Mol. Cell. Biol.* **9**, 5022–5033.
12. Cserjesi, P. and Olson, E.N. (1991) *Mol. Cell. Biol.* **11**, 4854–4862.
13. Lassar, A.B., Davis, R.L., Wright, W.E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D. and Weintraub, H. (1991) *Cell* **66**, 305–315.
14. Vandromme, M., Gauthier-Rouvière, C., Carnac, G., Lamb, N. and Fernandez, A. (1992) *J. Cell Biol.* **118**, 1489–1500.
15. Treisman, R. (1987) *EMBO J.* **6**, 2711–2717.
16. Boxer, L. M., Prywes, R., Roeder, R.G. and Kedes, L. (1989) *Mol. Cell. Biol.* **9**, 512–522.
17. Lee, T.-C., Chow, K.-L., Fang, P. and Schwartz, R.J. (1991) *Mol. Cell. Biol.* **11**, 5090–5100.
18. Sartorelli, V., Webster, K.A. and Kedes, L. (1990) *Genes Dev.* **4**, 1811–1822.
19. Mar, J. H. and Ordahl, C.P. (1990) *Mol. Cell. Biol.* **10**, 4271–4283.
20. Geisterfer-Lowrance, A.A.T., Kass, S., Tanigawa, G., Vosberg, H.-P., McKenna, W., Seidman, C.E. and Seidman, J.G. (1990) *Cell* **62**, 999–1006.
21. Tanigawa, G., Jarcho, J.A., Kass, S., Solomon, S.D., Vosberg, H.-P., Seidman, J.G. and Seidman, C.E. (1990) *Cell* **62**, 991–998.
22 Cribbs, L.L., Shimizu, N., Yockey, C.E., Levin, J.E., Jakovcic, S., Zak, R. and Umeda, P.K. (1989) *J. Biol. Chem.* **264**, 10672–10678.
23. Flink, I.L., Edwards, J.G., Bahl, J.J., Liew, C.-C., Sole, M. and Morkin, E. (1992) *J. Biol. Chem.* **267**, 9917–9924.
24. Thompson, W.R., Nadal-Ginard, B. and Mahdavi, V. (1991) *J. Biol. Chem.* **266**, 22678–22688.
25. Shimizu, N., Dizon, E. and Zak, R. (1992) *Mol. Cell. Biol.* **12**, 619–630.
26. Xiao, J.H., Davidson, I., Matthes, H., Garnier, J.-M. and Chambon, P. (1991) *Cell* **65**, 551–568.
27. Farrance, I.K.G., Mar, J.H. and Ordahl, C.P. (1992) *J. Biol. Chem.* **24**, 17234–17240.
28. Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) *Nucleic Acids Res.* **11**, 1475–1489.
29. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
31. Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
32. Silva, C.M., Tully, D.B., Petch, L.A., Jewell, C.M. and Cidlowski, J.A. (1987) *Proc. Natl. Acad. Sci. USA.* **84**, 1744–1748.
33. Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) *Biochemistry* **18**, 5294–5299.
34. Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159.
35. Wigler, M., Pellicer, A., Silverstein, S., Axel, R., Urlaub, G. and Chasin, L. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1373–1376.
36. Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982) *Mol. Cell. Biol.* **2**, 1044–1051.
37. Miller, J.H. (1972) Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. Bürglin, T.R. (1991) *Cell* **66**, 11–12.
39. Hwang, J.-J., Chambon, P. and Davidson, I. (1993) *EMBO J.* **12** 2337–2348.
40. Mirabito, P.M., Adams, T.H. and Timberlake, W.E. (1989) *Cell* **57**, 859–868.
41. Laloux, I., Dubois, E., Dewerchin, M. and Jacobs, E. (1990) *Mol. Cell. Biol.* **10**, 3541–3550.
42. Campbell, S., Inamdar, M., Rodrigues, V., Raghavan, V., Palazzolo, M. and Chovnick, A. (1992) *Genes Dev.* **6**, 367–379.
43. Ishiji, T., Lace, M.J., Parkkinen, S., Anderson, R.D., Haugen, T.H., Cripe, T.P., Xiao, J.-H., Davidson, I., Chambon, P. and Turek, L.P. (1992) *EMBO J.* **6**, 2271–2281.
44. Murre, C., McCaw, P.S., Vassin, H., Caudy, M., Jan, L.Y., Jan, Y.N., Cabrerra, C.V., Buskin, J.N., Hauschka, S.D., Lassar, A.B., Weintraub, H. and Baltimore, D. (1989) *Cell* **58**, 537–544.
45. Voss, J.W., Wilson, L. and Rosenfeld, M.G. (1991) *Genes Dev.* **5**, 1309–1320.
46. Blatt, C. and DePamphilis, M.L. (1993) *Nucleic Acids Res.* **21**, 747–748.