chor oligonucleotide (Operon, Alameda, CA) at 37°C for 2 hours, followed by heat inactivation at 70°C for 15 min, then digestion with Eco RI restriction endonuclease, and purification with QIAquick PCR kit (Qiagen, Valencia, CA). The ligated DNA (150 ng) was amplified by PCR with an upper primer composed of a subtelomeric sequence present internally to 11 out of the 12 *K. lactis* telomeres (*15*) and an Apa I restriction site (5′-GACCGGGCCCAGCAGGACCAAG-3′), and a lower primer complementary to the anchor primer and containing an Eag I restriction site (5′-CGACGCGGCCGCTTATTAACCCT-3′). PCR products were extracted from an agarose gel, cloned into a Bluescript vector, and sequenced.

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Promoter-Selective Properties of the TBP-Related Factor TRF1

Michael C. Holmes and Robert Tjian*

The TATA-binding protein (TBP)–related factor 1 (TRF1) is expressed in a tissue-restricted fashion during *Drosophila* embryogenesis and may serve as a promoter-specific recognition factor that can replace TBP in regulating transcription. However, bona fide target promoters that would preferentially respond to TRF1 have remained elusive. Polytene chromosome staining, chromatin immunoprecipitation, direct messenger RNA analysis, and transient cotransfection assays identified the *Drosophila* gene *tudor* as containing a TRF1-responsive promoter. Reconstituted in vitro transcription reactions and deoxyribonuclease I footprinting assays confirmed the ability of TRF1 to bind preferentially and direct transcription of the *tudor* gene from an alternate promoter. Thus, metazoans appear to have evolved gene-selective and tissue-specific components of the core transcription machinery to regulate gene expression.

Diverse mechanisms have evolved to regulate the spatial and temporal patterns of gene expression required for growth, differentiation, and response to environmental stimuli (*1*). Cell type–specific transcriptional activators that interact with enhancer DNA sequences to control programs of gene expression in metazoans have received much attention. In contrast, the general transcriptional apparatus has been viewed as a nonregulated "basal" component because the RNA polymerase II (Pol II) machinery was largely thought to be invariant in its composition or expression. However, these earlier studies did not anticipate the possibility that the Pol II machinery itself might display tissue-specific or gene-selective properties. In 1993, a novel TBP-related gene product, TRF1, was isolated from *Drosophila* and subsequently found to display properties expected of a cell type–specific TBP molecule (*2, 3*). In addition to being expressed in a tissue-restricted fashion, TRF1 was able to interact with TFIIA and TFIIB to form a Pol II preinitiation complex that accurately directs transcription in vitro. Polytene chromosome staining with an antibody to TRF1 revealed its association in vivo with a small subset of genes within the *Drosophila* genome. However, there was no evidence that TRF1 could differentially recognize distinct classes of promoters.

To identify promoters regulated by TRF1, we performed chromatin immunoprecipitation experiments with formaldehyde-treated SL2 cells (*4*). The *tudor* gene previously identified by TRF1 chromosome staining was specifically tested for TRF1 interaction. A 400–base pair (bp) fragment of the *tudor* promoter was probed by Southern hybridization with ^{32}P-labeled DNA prepared from the chromatin immunoprecipitations. As a control, we also probed a 1.7-kb fragment of the *Antennapedia* P1 promoter (AntP1). DNA sequences crosslinked and precipitated with TRF1 hybridized strongly to the *tudor* promoter fragment, whereas no signal was detected for the AntP1 fragment (Fig. 1, lanes 7 and 8). By contrast, DNA sequences coprecipitated with the transcription factor Adf-1 hybridized strongly to the AntP1 promoter but not the *tudor* promoter (Fig. 1, lanes 5 and 6). Adf-1 had been previously shown to regulate the expression of *Antennapedia* through the P1 promoter (*5*). Anti-TBP or control beads alone failed to precipitate either promoter fragment (Fig. 1, lanes 1 to 4). These studies taken together suggest that in vivo TRF1 associates selectively with DNA sequences within 500 bp of the *tudor* promoter.

To determine whether TRF1 could functionally modulate the expression of *tudor*, we

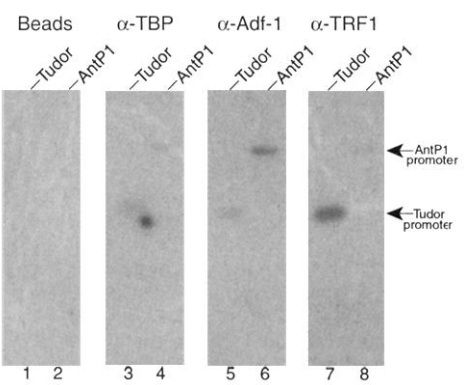

Fig. 1. TRF1 associates with the *tudor* core promoter in SL2 cells. DNA hybridization blots were probed with DNA isolated from chromatin immunoprecipitations of formaldehyde-treated SL2 cells. Each blot contained restriction fragments of the *tudor* and *Antennapedia* P1 (AntP1) promoters, which were separated by agarose gel electrophoresis and transferred onto Gene Screen Plus nylon membranes (NEN Life Science Products). Chromatin immunoprecipitations were carried out using antibodies to TBP (α-TBP), Adf-1 (α-Adf-1), or TRF1 (α-TRF1). As a negative control, mock immunoprecipitations were performed with protein A–Sepharose beads alone. On the α-TRF1 blot, the DNA crosslinked to TRF1 hybridized to the 400-bp *tudor* promoter fragment (lane 7), indicating that TRF1 binds a DNA sequence within 500 bp of the *tudor* core promoter in vivo. The DNA precipitated with TRF1 antibodies did not cross-react with the 1.7-kb AntP1 promoter fragment (lane 8). Arrows denote the positions of the *tudor* and AntP1 promoters on the blots. The DNA isolated in the Adf-1 immunoprecipitation hybridized to the AntP1 promoter fragment (lane 6) but not to the *tudor* promoter fragment (lane 5). The α-TBP and beads-only control immunoprecipitations (lanes 1 to 4) failed to efficiently precipitate either the *tudor* or AntP1 promoter fragments.

Howard Hughes Medical Institute, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA.

*To whom correspondence should be addressed. E-mail: jmlim@uclink4.berkeley.edu

established stable cell lines that overexpressed TRF1 under control of the inducible metallothionein promoter in SL2 cells (*6*). After a 6-hour induction with 400 μM $CuSO_4$, mRNA was collected from TRF1-expressing and control cells. Protein immunoblot assays confirmed that maximal TRF1 expression was achieved within 6 hours of $CuSO_4$ induction (Fig. 2A). The *tudor* gene was consistently up-regulated (by a factor of >2.5) by TRF1 expression, as determined by primer extension analysis (Fig. 2B). Interestingly, *tudor* transcription initiated from two start sites in vivo (*7*), but only start site 2 was stimulated efficiently by TRF1. RNA hybridizations confirmed that control genes (*hsp83*) were unchanged between the two mRNA populations (*7*). Induction of TRF1 in SL2 cells revealed a number of potential TRF1 target genes, including the *tudor* gene that was identified in both the chromatin staining and immunoprecipitation experiments, which suggested that TRF1 mediates *tudor* expression.

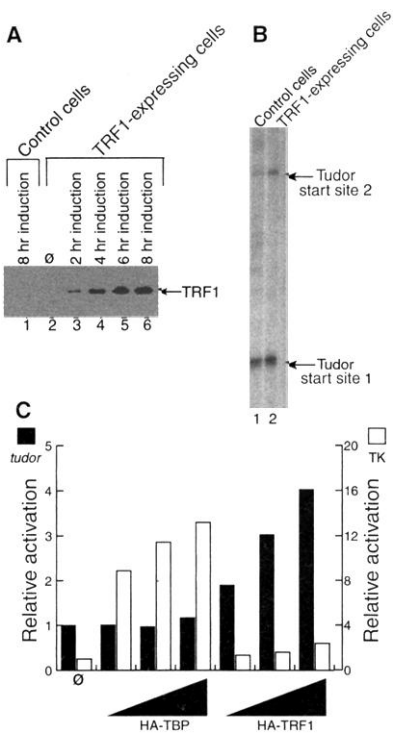

Fig. 2. The *tudor* promoter is induced in transfected SL2 cells expressing TRF1. Messenger RNA was purified from a cell line expressing TRF1 under the metallothionein promoter and a control cell line transfected with only the metallothionein promoter vector. (**A**) TRF1 induction was monitored by protein immunoblot assays using monoclonal antibodies to the hemagglutinin epitope tag. Cell lysates were prepared from both the TRF1 expressing cells and the control cells collected at different time points; ø refers to cells collected just before induction. Maximal TRF1 expression was observed within 6 hours of $CuSO_4$ induction. (**B**) Messenger RNA was isolated using oligo(dT) cellulose (Ambion) and analyzed by primer extension and RNA hybridization blots. Arrows indicate the positions of start sites 1 and 2 on the gel. The levels of transcription initiated at each of the two start sites are compared between the cell lines (lanes 1 and 2). The mRNA collected from the cell line expressing TRF1 shows an increase in the level of *tudor* transcription (lane 2). However, the relative induction of start site 2 is higher than that of start site 1. (**C**) Transient transfections with the *tudor*-luciferase reporter show a dosage-dependent stimulation by TRF1 on the *tudor* promoter with no effect by TBP (black bars, left *y* axis) (*14*). Conversely, the HSV TK promoter is strongly stimulated by cotransfecting in TBP, whereas TRF1 poorly stimulated the HSV TK promoter (white bars, right *y* axis).

To obtain more direct evidence, we fused the *tudor* promoter (–322 to +100) to a luciferase reporter and performed transient transfection experiments in SL2 cells expressing either TRF1 or TBP under control of the actin 5C promoter. When the *tudor* promoter was cotransfected with TRF1, there was a dose-dependent activation response resulting in a substantial stimulation of the promoter, whereas cotransfection of the *tudor* promoter with similar amounts of the TBP expression construct had little or no effect on the promoter (Fig. 2C). The well-documented TBP-responsive herpes simplex virus (HSV) thymidine kinase (TK) promoter was strongly stimulated by increasing amounts of TBP, whereas TRF1 stimulated the TK promoter poorly (Fig. 2C) (*8*). These results suggest that in SL2 cells the *tudor* promoter is preferentially stimulated by TRF1 relative to TBP, whereas the TK promoter is preferentially stimulated by TBP. This differential promoter selectivity of TRF1 over TBP on the *tudor* promoter is consistent with the notion that TRF1 may function as a promoter-selective transcription factor.

To initiate a more mechanistic analysis of TRF1, we carried out in vitro transcription with the *tudor* promoter. A combination of recombinant basal factors and components purified from *Drosophila* embryo extracts was used to reconstitute transcription under conditions that were completely dependent on the addition of TFIID, recombinant TBP (rTBP), or rTRF1 (Fig. 3A, lane 1). Adding TFIID or rTBP stimulated transcription from start site 1 but had little effect on start site 2 (Fig. 3A, lanes 2 to 4 and 5 to 8). In marked contrast, rTRF1 strongly stimulated transcription from start site 2 but had little effect on start site 1 (Fig. 3A, lanes 9 to 12). A construct containing only start site 2 was compared with the distal promoter of the alcohol dehydrogenase (*adh*) gene (*5*). Once again, rTRF1 strongly stimulated transcription from *tudor* start site 2, whereas rTBP had little or no effect (Fig. 3B, lanes 11 to 14 and 15 to 18). By contrast, the *adh* promoter was strongly stimulated by rTBP, whereas the ability of rTRF1 to

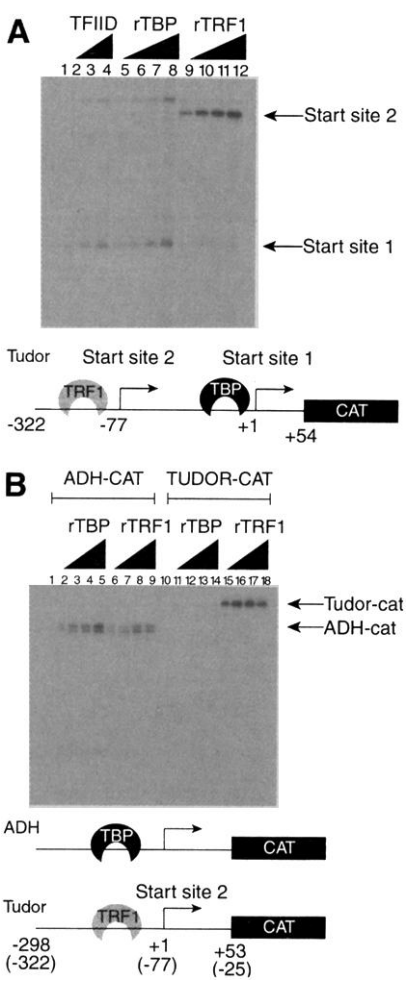

Fig. 3. In vitro, TRF1 stimulates transcription of *tudor* using an alternative promoter. Two in vivo start sites 77 bp apart, designated start sites 1 and 2, were mapped for the *tudor* promoter using primer extension. (**A**) In vitro transcription assays using recombinant and purified basal factors from embryo nuclear extracts were used to reconstitute transcription in vitro on the *tudor* promoter (*15*). As shown in lane 1, the reactions are completely dependent on addition of TFIID, rTBP, or rTRF1. Upon addition of either TFIID (0.5, 1, or 2 μl of a partially purified fraction) or rTBP (5, 10, 20, or 40 ng) (lanes 2 to 8), transcription is stimulated from start site 1. If rTRF1 (5, 10, 20, or 40 ng) is added (lanes 9 to 12), transcription is initiated from start site 2. Arrows indicate the location of products initiated from the two tandem promoters (start sites 1 and 2). (**B**) In vitro transcription reactions were carried out comparing the relative activities of rTRF1 (5, 10, 20, and 40 ng) and rTBP (5, 10, 20, and 40 ng) on the *tudor* site 2 promoter and the distal promoter of the *adh* gene. On the *tudor* site 2 promoter, there was no effect by rTBP (lanes 11 to 14), whereas strong stimulation was observed by the addition of rTRF (lanes 15 to 18). The *adh* promoter was preferentially stimulated by rTBP (lanes 2 to 5) with rTRF showing weak stimulation (lanes 6 to 9), demonstrating the promoter-selective activity of TRF1. The position of the primer extension products for each promoter is marked by arrows. The numbers in parentheses under the *tudor* site 2 promoter construct indicate the nucleotide positions relative to start site 1 shown in Fig. 4A.

direct transcription was rather attenuated (Fig. 3B, lanes 2 to 5 and 6 to 9), consistent with our previous finding that TRF1 can only partially substitute for TBP in vitro (*3*). As an additional control, this experiment was repeated with α-amanitin added to a final concentration of 1 μg/ml to confirm that the stimulation is dependent on RNA Pol II (*7*). These studies support the conclusion that TRF1 and TBP/TFIID exhibit differential promoter selectivity in vitro and suggest that TRF1 preferentially recognizes and mediates transcription from *tudor* promoter site 2.

To biochemically characterize the basis for this promoter selectivity, we carried out deoxyribonuclease I (DNase I) footprinting experiments with the *tudor* promoter site 2 region. When purified rTRF1 was incubated with the promoter DNA fragment, little or no specific binding was apparent (Fig. 4A, lane 9). However, upon addition of rTFIIA and rTFIIB, we observed a clear footprint that spans nucleotides −22 to −33 in relation to start site 2 (Fig. 4A, lanes 10 and 11). By contrast, there was no binding of TBP to this region, even upon addition of rTFIIA or rTFIIB (Fig. 4A, lanes 4 to 6), consistent with the finding that TBP inefficiently mediated transcription from *tudor* site 2. This TRF1 binding region was designated a TC-box, analogous to the TATA-box, because of the TC-rich nature and relative upstream position (−25) of this sequence.

To further substantiate the role of the TC-box, we generated four mutant constructs and tested their ability to support TRF1-dependent transcription in vitro (Fig. 4B). Transcription directed by the mut 1, 2, and 4 templates was completely abolished (Fig. 4B, lanes 4 to 7, 10, and 11), whereas the mut 3 template (Fig. 4B, lanes 8 and 9) supported transcription at one-fourth of the wild-type level (Fig. 4B, lanes 2 and 3). Mut 1 and mut 2 also failed to show any binding to TRF1 in footprinting assays (*7*). These studies suggest that the promoter selectivity of TRF1 may be at least partly achieved through its differential recognition of TC-boxes.

Using multiple in vivo and in vitro assays, we have identified a candidate *Drosophila* gene, *tudor*, that contains two tandem promoters, one of which is targeted by TRF1. In vitro, TRF1 preferentially nucleated the recruitment of the basal machinery to an alternate promoter upstream of the start site used by TBP/TFIID. This *tudor* site 2 start position contains an upstream TC-box element that is selectively recognized and bound by TRF1 but fails to bind TBP, whereas site 1 responded to TBP/TFIID but not TRF1. Such an arrangement of tandem promoters provides a mechanism by which TRF1 could substitute for TBP in regulating specific subsets of genes to establish cell type–specific gene expression.

The ability of TRF1 to discriminate between different core promoter sequences may not be solely due to its intrinsic DNA recognition properties. TRF1 has been found complexed with other proteins [designated neuronal TRF1-associated factors (nTAFs)], and therefore promoter specificity may depend in part on these associated factors (*3*). The other likely possibility is that promoter-specific transcriptional activators may help recruit TRF1 to the subset of genes it regulates. Thus far, partially purified TRF1-containing complexes have not been transcriptionally active, and our efforts to further characterize the putative nTAFs have been unsuccessful. Recent evidence suggests that TRF1 is also involved with RNA Pol III transcription in *Drosophila*, consistent with our previous polytene chromosome staining studies that revealed multiple tRNA genes associated with TRF1 (*3, 9*). However, a more extensive analysis of how TRF1 functions both in vivo and in vitro would be required to determine the distinct modes by which TRF1 may modulate the expression of different classes of genes. Future efforts to identify TRF1-responsive genes would benefit from an analysis of high-density microarrays containing several thousand *Drosophila* cDNAs. A preliminary but incomplete gene expression array analysis suggests that ~5% of *Drosophila* mRNAs become up-regulated within 6 hours of TRF1 induction in SL2 cells (*10*).

TRF1 is the founding member of a family of TBP-related molecules. A more distantly related TBP-like factor (designated TRF2, TLF, or TRP) was recently identified in *Caenorhabditis elegans*, *Drosophila*, mouse, and humans, but not in yeast (*11*). Curiously, TRF1 has thus far only been found in *Drosophila*. In addition to cell type–restricted TBP-related factors, studies have also identified the existence of tissue-specific TAF_{II}'s, including $TAF_{II}105$ (which is associated with TFIID in B lymphocytes) as well as *cannonball* and *no hitter*, two spermatocyte-specific TAFs related to *Drosophila* $TAF_{II}80$ and $TAF_{II}110$, respectively (*12*). The existence of cell type–specific components of the general machinery is reminiscent of bacterial sigma factors that are required for assembling different RNA polymerase holoenzymes dedicated to the selective transcription of distinct classes of bacterial genes (*13*). We imagine that specialized components of the core machinery, such as cell type–specific TAFs and TRFs, may provide multicellular organisms with additional levels of specificity and control to execute the elaborate programs of gene expression required during growth, differentiation, and development.

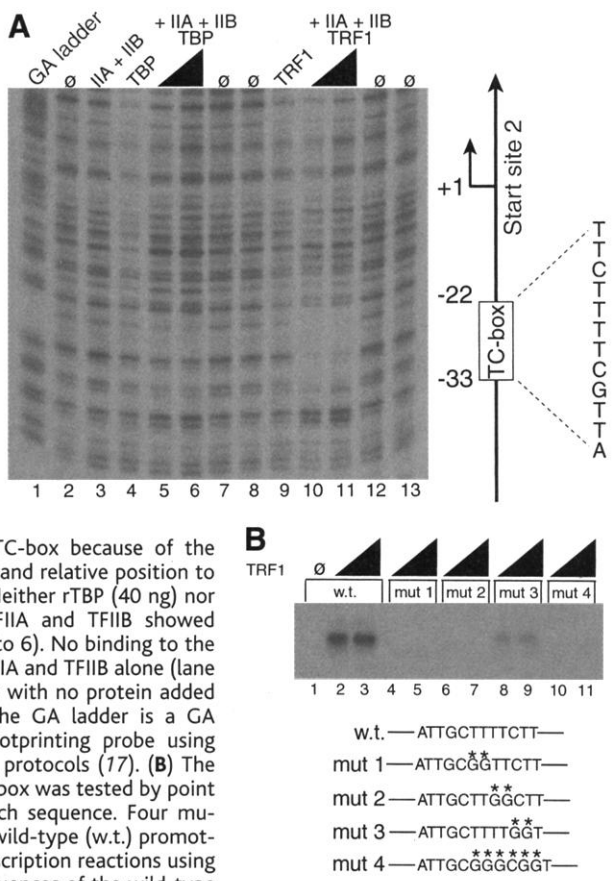

Fig. 4. TRF1 binds specifically to the TC-box upstream of *tudor* start site 2. (**A**) The binding of TRF1 to the upstream promoter region of *tudor* start site 2 was analyzed by DNase I footprinting as described (*16*). The proteins were incubated with the template for 30 min at room temperature and then digested with DNase I. When rTRF1 (40 ng) was added to the template (lane 9), no specific binding was readily apparent. Upon addition of TFIIA (50 ng) and TFIIB (30 ng) with rTRF1 (20 and 40 ng) (lanes 10 and 11), a clear footprint spanning the –22 to –33 region of the *tudor* site 2 promoter was observed. This element was designated the TC-box because of the TC-rich nature of the sequence and relative position to the start site of transcription. Neither rTBP (40 ng) nor rTBP (20 and 40 ng) with TFIIA and TFIIB showed binding to the TC-box (lanes 4 to 6). No binding to the promoter was detected with TFIIA and TFIIB alone (lane 3). ø refers to control reactions with no protein added (lanes 2, 7, 8, 12, and 13). The GA ladder is a GA cleavage reaction with the footprinting probe using Maxam and Gilbert sequencing protocols (*17*). (**B**) The dependence of TRF1 on the TC-box was tested by point mutation analysis of the TC-rich sequence. Four mutants were compared with the wild-type (w.t.) promoter in reconstituted in vitro transcription reactions using 20 and 40 ng of rTRF1. The sequences of the wild-type and mutant constructs are listed (an asterisk denotes the nucleotides mutated). TRF1-dependent transcription was completely abolished from the mutated mut 1, 2, and 4 templates (lanes 4 to 7, 10, and 11), whereas the mut 3 template weakly supported TRF1-dependent transcription (lanes 8 and 9) at one-fourth of the wild-type level (lanes 2 and 3).

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15. Recombinant TBP, TFIIA, and TFIIB were purified as described [S. K. Hansen and R. Tjian, *Cell* **82**, 565 (1995); B. F. Pugh and R. Tjian, *Genes Dev.* **5**, 1935 (1991)]. TRF1 was expressed recombinantly in *Escherichia coli* and purified as described (*5*). TFIID, TFIIE, TFIIF, TFIIH, and RNA Pol II were purified from nuclear extracts derived from 0- to 12-hour *Drosophila* embryos. Nuclear extracts were prepared as described [U. Heberlein and R. Tjian, *Nature* **331**, 410 (1988)], dialyzed into 0.1 M KCl HEMG buffer [25 mM Hepes (pH 7.6), 12.5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1 mM PMSF, and 1 mM sodium metabisulfite], and loaded onto a 40-ml Poros 20 HE1 column. The basal factors were stepped off with 0.4 M NaCl HEMG buffer and loaded onto a 520-ml Sephacryl-300 column (S300, Pharmacia) equilibrated in 0.1 M NaCl HEMG buffer. The proteins were resolved isocratically and the fractions assayed for transcriptional activity. TFIID activity was isolated from fractions corresponding to the void volume of the S300 column. Several fractions eluting after the void volume and TFIID were tested and found to contain the TFIIE, TFIIF, TFIIH, and RNA Pol II activities used in the experiments for this study. TFIID was further purified by directly loading the S300 fractions containing TFIID activity onto an 8-ml Mono-Q HR 10/10 column (Pharmacia). TFIID was eluted from the column with a 25-column volume gradient from 0.1 to 0.5 M NaCl in HEMG buffer. TFIID activity eluted between 0.25 and 0.3 M NaCl.
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Requirement of JNK for Stress-Induced Activation of the Cytochrome c–Mediated Death Pathway

Cathy Tournier,[1] Patricia Hess,[1] Derek D. Yang,[2]* Jie Xu,[2] Tod K. Turner,[3] Anjaruwee Nimnual,[4] Dafna Bar-Sagi,[4] Stephen N. Jones,[3] Richard A. Flavell,[2] Roger J. Davis[1]†

The c-Jun NH_2-terminal kinase (JNK) is activated when cells are exposed to ultraviolet (UV) radiation. However, the functional consequence of JNK activation in UV-irradiated cells has not been established. It is shown here that JNK is required for UV-induced apoptosis in primary murine embryonic fibroblasts. Fibroblasts with simultaneous targeted disruptions of all the functional *Jnk* genes were protected against UV-stimulated apoptosis. The absence of JNK caused a defect in the mitochondrial death signaling pathway, including the failure to release cytochrome c. These data indicate that mitochondria are influenced by proapoptotic signal transduction through the JNK pathway.

The development and maintenance of healthy tissues involves apoptosis, a program of physiologically regulated cell death (*1*). Dysregulated apoptosis contributes to many pathologies, including tumor promotion, autoimmune and immunodeficiency diseases, and neurodegenerative disorders (*2*). Therefore, signaling pathways that trigger apoptosis are of intense interest. The c-Jun NH_2-terminal kinase (JNK) signaling pathway is essential for neuronal apoptosis in response to excitotoxic stress (*3*). However, the role of JNK in the apoptotic responses of other cell types is unclear. The goal of this study was to define the requirement for JNK in apoptosis of primary murine embryonic fibroblasts (MEF).

MEF were prepared from wild-type (WT) embryos and mutant embryos in which the *Jnk* genes were disrupted (*3, 4*). WT MEF expressed large amounts of 46-kD JNK1 and 55-kD JNK2 isoforms and small amounts of 55-kD JNK1 and 46-kD JNK2 isoforms (Fig. 1A). The neuronal JNK isoform (JNK3) was not detected (*5*). The compound mutant $Jnk1^{-/-}Jnk2^{-/-}$ MEF did not express JNK1 or JNK2. These data indicated that $Jnk1^{-/-}$ $Jnk2^{-/-}$ MEF may lack a functional JNK signal transduction pathway. This conclusion was confirmed by analysis of JNK activity in MEF exposed to ultraviolet (UV) radiation (Fig. 1B) or treated with fetal bovine serum (Fig. 1C). Disruption of the *Jnk1* and *Jnk2* genes did not alter expression of related mitogen-activated protein kinases (MAPK), ERK and p38, or the JNK activators MKK4 and MKK7 (Fig. 1A). The MKK4 and MKK7

[1]Howard Hughes Medical Institute, Program in Molecular Medicine, Department of Biochemistry & Molecular Biology, University of Massachusetts Medical School, Worcester, MA 01605, USA. [2]Howard Hughes Medical Institute, Section of Immunobiology, Yale University School of Medicine, New Haven, CT 06520, USA. [3]Department of Cell Biology, University of Massachusetts Medical School, Worcester, MA 01605, USA. [4]Department of Molecular Genetics and Microbiology, State University of New York at Stony Brook, Stony Brook, NY 11794, USA.

*Present address: Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285, USA.

†To whom correspondence should be addressed. E-mail: Roger.Davis@umassmed.edu