diolabeled Chemicals). [^{3}H]Mannose labeling was done in glucose-free and L-glutamine–free medium supplemented with 5% dialyzed fetal bovine serum, dextrose (0.5 mg/ml), sodium pyruvate (1.1 mg/ml), antibiotics, nucleosides, and nonessential amino acids for 18 to 24 hours at 37°C. CD1d1 and Db-sol in the culture supernatant were purified with His-Trap Ni–Sepharose and B22-249–coupled protein A–Sepharose columns, respectively, after preclearing with Hi-Trap protein A–Sepharose according to the manufacturer (Pharmacia Biotech). Radioactivity in each fraction was monitored with a scintillation counter (Beckman). [^{3}H]Mannose-labeled, sCD1d1 and Db-sol–associated ligands were separated from the heavy and light chains by Microcon-10 (Amicon) filtration to specifically monitor GPI-associated radioactivity.

26. sCD1d1 and H-2D^b (about 4 μM), in triplicate, were mixed with 1.8 μCi of L-α-[*myo*-inositol-2-^{3}H]PI (11 Ci/mmol; DuPont-NEN) in 100 μl of phosphate-buffered saline at 37°C for about 18 hours. sCD1d1- and H-2D^b–bound [^{3}H]PI were separated from free [^{3}H]PI by Microcon-10 filtration, and radioactivity in the retained solution was measured in a scintillation counter. H-2D^b reconstituted in vitro from heavy and light chains produced in *Escherichia coli* and with a H-2D^b–binding peptide, Gly-Ala-Ile-Ser-Asn-Met-Tyr-Ala-Met, derived from glutamic acid dehydrogenase was used as the control for binding specificity. The in vitro reconstituted H-2D^b was generously provided by E. Palmieri and S. G. Nathenson. For Scatchard analysis, various concentrations of purified sCD1d1, in duplicate, were mixed with 1.8 μCi of [^{3}H]PI (~1.6 μM) in 100 μl of 20 mM phosphate buffer, pH 7.4. After incubation at 37°C for ~18 hours, sCD1d1-[^{3}H]PI complexes were separated from free [^{3}H]PI by Microcon-10 filtration, and radioactivity in the retained solution was measured.
27. Clearly GPI is the major ligand identified under the conditions described here for the isolation, RP-HPLC fractionation, and MALDI-MS analysis (Fig. 1). To determine whether GPI is the major or the only natural ligand of CD1d1, we estimated the percent of CD1d1 occupied by GPI from the ratio of [^{3}H]mannose-labeled heavy chain to [^{3}H]mannose-labeled GPI. Considering that D-[2-^{3}H]mannose converts mostly to D-[2-^{3}H]fucose and rarely to other sugars (*33*), there are about seven times as many mannoses and fucoses in the heavy chain as in GPI (*17*). Thus ~65% of CD1d1 is occupied by GPI before accounting for losses incurred during the purification steps. Assuming 65 to 70% recovery of the ligand [based on peptide recoveries from class I molecules (*15, 34*)], then >90% of CD1d1 is occupied by GPI.
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A Screen for Genes Induced in the Suprachiasmatic Nucleus by Light

Mary E. Morris, N. Viswanathan, Sandra Kuhlman, Fred C. Davis, Charles J. Weitz*

The mechanism by which mammalian circadian clocks are entrained to light-dark cycles is unknown. The clock that drives behavioral rhythms is located in the suprachiasmatic nucleus (SCN) of the brain, and entrainment is thought to require induction of genes in the SCN by light. A complementary DNA subtraction method based on genomic representational difference analysis was developed to identify such genes without making assumptions about their nature. Four clones corresponded to genes induced specifically in the SCN by light, all of which showed gating of induction by the circadian clock. Among these genes are c-*fos* and *nur77*, two of the five early-response genes known to be induced in the SCN by light, and *egr-3*, a zinc finger transcription factor not previously identified in the SCN. In contrast to known examples, *egr-3* induction by light is restricted to the ventral SCN, a structure implicated in entrainment.

Daily rhythms of biological activity, manifested by forms as diverse as cyanobacteria, fungi, plants, and animals, are driven by self-sustaining, endogenous oscillators called circadian clocks (*1*), which typically run with an intrinsic period that is close to, but not exactly, 24 hours. Under natural conditions, circadian clocks become precisely entrained to the 24-hour light-dark cycle because exposure to light at certain times induces a phase shift of the clock. Entrainment to light-dark cycles ensures that the clock adopts a specific and stable phase relation to the natural day, setting the clock to local time and enabling the organism to anticipate daily environmental events (*2*).

M. E. Morris and C. J. Weitz, Department of Neurobiology, Harvard Medical School, Boston, MA 02115, USA. N. Viswanathan, S. Kuhlman, F. C. Davis, Department of Biology, Northeastern University, Boston, MA 02115, USA.

*To whom correspondence should be addressed.

In mammals, the circadian clock that drives daily rhythms of behavioral activity is located within the SCN of the hypothalamus (*3*). Entrainment of the clock to light-dark cycles is mediated by photoreceptors in the retina (*4*), and light information is conveyed directly from the retina to the SCN by the retinohypothalamic tract (*5*). Although the molecular basis of entrainment to light-dark cycles in mammals is unknown, the process likely involves light- and clock-dependent transcriptional regulation within the SCN (*6*). When a rodent kept in constant darkness is exposed to a brief light pulse during the subjective night, a time when the clock responds to light with a phase shift, five known early-response genes—c-*fos*, *fos-B*, *jun-B*, *zif268* (NGFI-A), and *nur77* (NGFI-B)—are specifically induced within the SCN (*7*). The genes are not induced by exposure to light during the subjective day, a time when the clock is not phase-shifted by light. Together, these and related experiments (*8*) strongly suggest that induction of genes in the SCN by light is an intermediate step in a pathway mediating entrainment of the clock to light-dark cycles. They further suggest that gating of this induction by the clock contributes to the restriction of phase-shifting by light to certain times, a feature that is essential for achieving stable entrainment (*9*).

To discover potential components of the entrainment pathway, we sought to identify genes induced in the SCN by light without making assumptions about their nature. We developed a cDNA subtraction method based on genomic representational difference analysis (RDA) (*10*) and carried out subtractions as follows: Syrian hamsters were entrained to a light-dark cycle for 3 weeks and then placed in constant dim light (<1 lux) for 1 week (*11*). Animals were then assigned to either of two equal groups for light treatment (30 min, 250 lux) or sham treatment (similar handling, <1 lux) at circadian time (CT) 19, a time during subjective night that is optimal for a phase advance by light. At the end of the treatment, SCNs were removed by micropunch from 40 animals in each group. The remaining animals served as controls (Fig. 1). As expected, light treatment resulted in a phase advance (Fig. 1A), whereas sham treatment resulted in little or no phase shift (Fig. 1B).

Starting with 1 μg of polyadenylated RNA from each of the two groups of 40 SCN tissue punches, we generated cDNA representations (*12*). Each RDA experiment (*13*) was performed so as to identify genes induced by light ("forward") and genes suppressed by light ("reverse") (*13*), the latter used here solely as a control. After three rounds of RDA, polymerase chain reaction (PCR) products were cloned and

recombinant plasmids were chosen at random for screening by differential hybridization (*14*). Duplicate Southern (DNA) blots were prepared for sets of plasmids, and one blot from each duplicate was hybridized to ^{32}P-labeled cDNA derived from SCNs of light-treated animals and the other to a comparable probe derived from SCNs of sham-treated animals; these are denoted +light and –light probes, respectively (Fig. 2A). Most inserts showed equivalent hybridization to the two probes, some showed no detectable hybridization to either probe, and some showed stronger hybridization to the +light probe than to the –light probe (asterisks in Fig. 2A). Of 792 RDA clones tested, 101 showed differential hybridization to the +light probe. After culling likely artifacts (*15*), sequencing and cross-hybridization indicated that the remaining 60 differentially hybridizing inserts corresponded to seven different clones. Notably, we obtained c-*fos* and *nur77*, two of the five early-response genes known to be induced in the SCN by light.

To exclude a fluke of sampling as the explanation for this result, we examined whether differentially hybridizing fragments had become progressively enriched through the RDA procedure (Fig. 2B). With a full-length c-*fos* probe, several PCR products showed enrichment at each stage and were observed only in the forward subtractions (Fig. 2B, DP1, DP2, and DP3, + lanes). For a probe corresponding to one of our differentially hybridizing inserts (designated rda-7), a PCR product corresponding in size to the rda-7 insert showed progressive enrichment and was observed only in the forward subtractions. Phosphorimaging indicated that c-*fos* was enriched by a factor of ~250 and rda-7 was enriched by a factor of >3000.

Next, we tested the in vivo regulation of genes corresponding to the differentially hybridizing RDA clones (Fig. 3) (*16*). A c-*fos* antisense riboprobe gave the expected results, showing specific c-*fos* induction in the SCN by light at CT 19 and CT 14 but

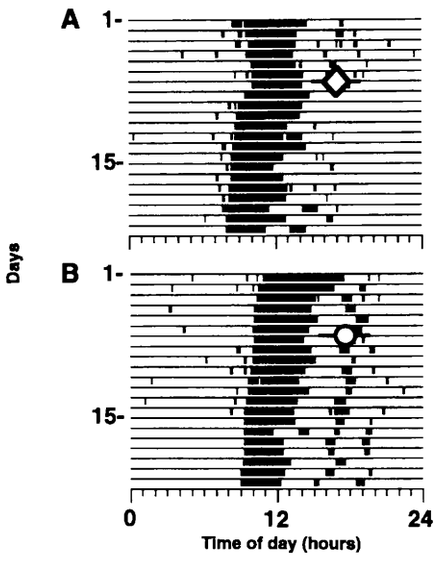

Fig. 1. Examples of controls confirming that the light or sham treatments had the expected effect on the phase of the circadian clock. Shown are spontaneous wheel-running activity records of two hamsters in constant dim light. Successive days are represented by horizontal lines, and the 24 hours within each day are represented on the *x* axis. Tick marks on horizontal lines represent bouts of spontaneous wheel-running activity. (**A**) Record from a hamster receiving a 30-min light pulse at the indicated time (diamond), corresponding to CT 19, as determined from the animal's spontaneous activity rhythm (not from the scale on the *x* axis). On the days after the light pulse, the shift to an earlier daily onset of wheel-running activity marks a phase advance, calculated here to be +1.10 hours (*2*). The mean from two light-treated hamsters was +1.23 hours. (**B**) Record from a hamster receiving a 30-min sham treatment at the indicated time (circle), corresponding to CT 19, as determined from the animal's spontaneous activity rhythm. The calculated phase shift was –0.04 hours; the mean from two sham-treated hamsters was –0.07 hours.

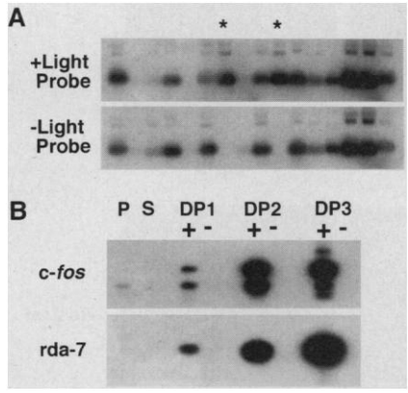

Fig. 2. (**A**) Example of initial characterization of RDA clones by differential hybridization. Duplicate Southern blots show hybridization of RDA clones to ^{32}P-labeled cDNA derived from SCNs of light-treated animals (+light probe) or to a comparable probe from SCNs of sham-treated animals (–light probe). Asterisks mark two inserts showing stronger hybridization to the +light probe; these are two copies of rda-7 (see text). (**B**) Progressive enrichment of differentially hybridizing RDA clones through the RDA procedure. Southern blots of cDNA (150 ng) from each stage of RDA were probed with full-length c-*fos* cDNA or rda-7 cDNA, as indicated. P, initial cDNA representation derived from SCNs of hamsters receiving light treatment; S, initial cDNA representation derived from SCNs of hamsters receiving sham treatment; DP1, DP2, and DP3, difference products obtained after one, two, and three rounds of RDA subtraction, respectively (+, forward subtraction; –, reverse subtraction) (*13*).

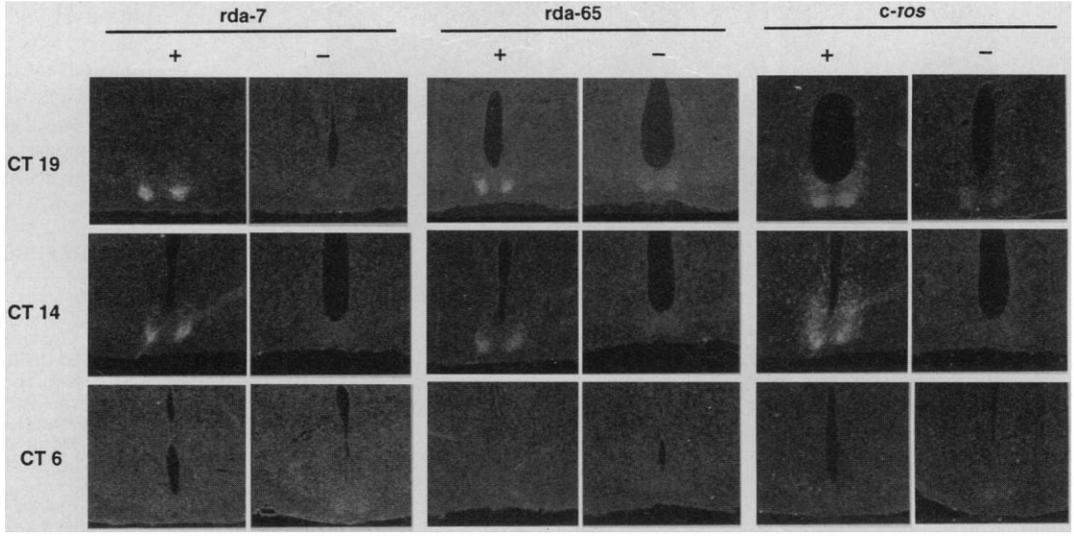

Fig. 3. In vivo regulation of transcripts corresponding to RDA clones; induction in the SCN by light and gating by the circadian clock. Shown are the results of in situ hybridization of antisense riboprobes (rda-7, rda-65, or c-*fos*) to coronal brain sections from hamsters subjected to a light (+) or sham (–) treatment at CT 19, 14, or 6. Variations in apparent size of the third ventricle are artifacts of tissue preparation.

not at CT 6; this reflects gating of induction by the circadian clock. Of the five RDA candidate clones, two, rda-7 and rda-65, detected transcripts showing specific induction in the SCN by light and gating by the circadian clock, much like that of c-*fos*, but with a cellular distribution within the SCN that appeared to differ from that of c-*fos*. The anatomical patterns and time courses of induction at CT 19 detected by the rda-7 and rda-65 riboprobes appeared identical; induction peaked during the first 30 min after the onset of exposure to light, became moderate at 2 hours, and returned to baseline by 4 hours (*17*). Little or no hybridization to the rda-7 and rda-65 riboprobes was detected in SCN sections from hamsters kept in constant dim light and killed at CT 2, 8, 14, or 20 (*17*); this result suggested that the transcripts do not contribute to the mechanism of the clock beyond a possible role in entrainment. As with c-*fos*, we detected no other prominent sites of induction in the brain by light, and sense riboprobe controls showed only background hybridization that did not differ between light- or sham-treated animals (*17*). Riboprobes from the remaining three candidate RDA clones did not reveal light-induced or clock-regulated genes.

Database searches revealed that rda-7 and rda-65 made significant sequence matches (*18*), respectively, to different parts of the 3′ untranslated region of human *egr-3*, an early-response gene encoding a zinc finger transcription factor (*19*). Further analysis of hamster cDNA and genomic clones indicated that rda-7 and rda-65 were both derived from the 3′ untranslated region of *egr-3*. Although not previously known to be induced in the SCN, *egr-3* is induced in various brain regions in response to stress or after focal brain injury (*20*). In the SCN, it is likely that *egr-3* participates in the transcriptional regulation of genes in response to retinal input, as has been proposed for c-*fos* (*6*).

Light-induced c-*fos* and *egr-3* transcripts consistently appeared to exhibit different anatomical distributions within the SCN (Fig. 3). To exclude animal-to-animal variation as the source of this difference, we examined c-*fos* and *egr-3* induction (Fig. 4A) or c-*fos*, *jun-B*, and *egr-3* induction (Fig. 4B) within SCNs of individual light-treated hamsters. As expected, c-*fos* induction was observed in both the dorsal and ventral regions of the SCN, as was *jun-B* induction. In contrast, *egr-3* induction was restricted to the ventral core of the SCNs. In addition, c-*fos* and *jun-B* induction were observed throughout the rostrocaudal extent of the SCN, whereas *egr-3* induction was observed only in the central part of this rostrocaudal extent. The ventral SCN structure in which *egr-3* is induced by light resembles that stained by antisera to calbindin-D_{28K} (*21*) or substance P (*22*).

The distinct cellular distribution of light-induced *egr-3* transcripts, as compared with light-induced c-*fos* and *jun-B* transcripts, indicates that the SCN responds to light in a complex fashion with overlapping but distinct regions activated in parallel. This divided response of the SCN to light could arise from an anatomically divided projection from the retina to the SCN, as suggested by electrical stimulation experiments (*23*), or from functional specialization of retinorecipient SCN cells, as suggested by the complex expression patterns of neurochemical markers (*22*). Pharmacological experiments have shown that retinal excitatory transmission to the ventral SCN is specifically required for circadian phase-shifting by light (*24*). Induction of *egr-3* itself, or activation of the ventral SCN structure that its induction by light reveals, could account for this requirement.

Note added in proof: Like *egr-3*, induction of the mouse *per1* gene by light shows a restriction to the ventral SCN (*25*).

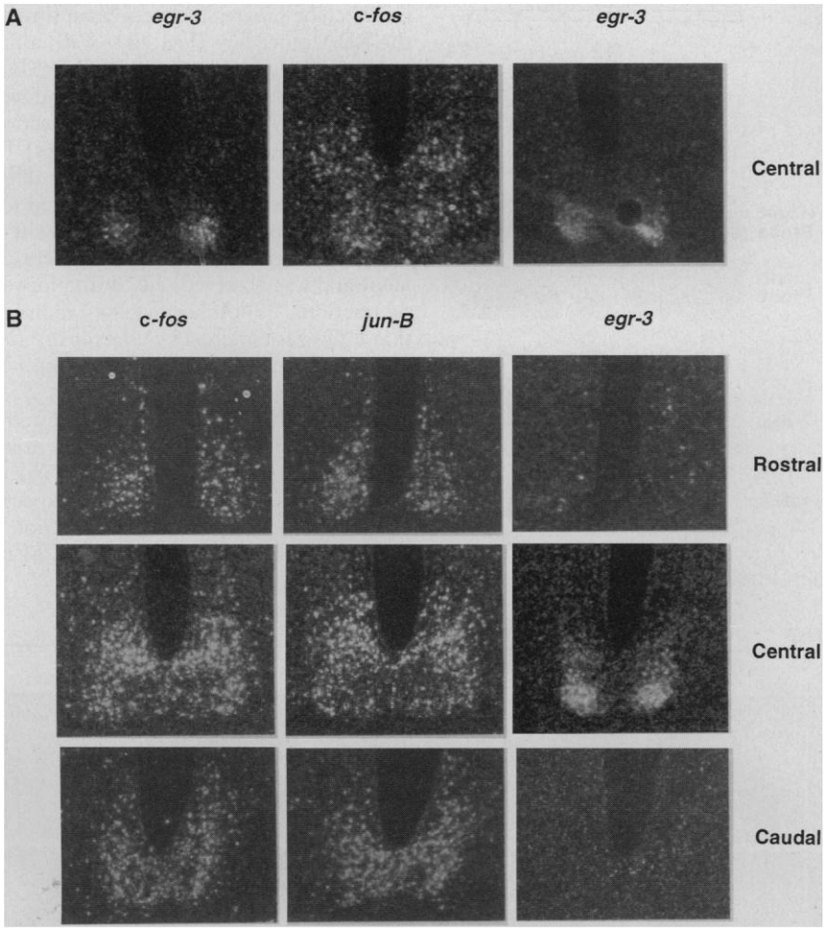

Fig. 4. Different cellular distribution within the SCN of light-induced *egr-3* transcripts as compared with light-induced c-*fos* and *jun-B* transcripts. Images show central sections (at the approximate center of the rostrocaudal extent of the SCN) or rostral and caudal margins of the SCN, as indicated at the right. (**A**) Neighboring coronal brain sections from a light-treated hamster hybridized, respectively, to *egr-3* (rda-7) or c-*fos* antisense riboprobes, as indicated. The right SCN in the right panel is distorted by an artifact of tissue preparation. (**B**) Sets of three neighboring coronal brain sections from a different light-treated hamster, each set taken from a different rostrocaudal level. Within each set, the three sections were hybridized, respectively, to c-*fos*, *jun-B*, or *egr-3* (rda-7) antisense riboprobes, as indicated.

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11. Eighty-four adult male Syrian hamsters (Charles River Laboratories) were entrained to a light-dark cycle (14 hours light, 10 hours dark) for 3 weeks. The hamsters were then transferred to constant dim light (<1 lux) for 7 days, and circadian phases were estimated from spontaneous running-wheel activity (*2*). At CT 19 on the seventh day, half of the hamsters received a light treatment (250 lux, 25 to 35 min), and the other half received a sham treatment (similar handling, but light <1 lux). At the end of the treatment, brains were removed (40 from each group) and placed in phosphate-buffered saline (PBS, 4°C) for 30 s, and a 1.5-mm-thick coronal slice containing the SCN was cut (Stoelting tissue slicer) and transferred to cold PBS. A 1-mm tissue punch (Fine Science Tools USA) containing the SCN was taken, frozen on dry ice, and stored (–70°C). The time between decapitation and freezing of dissected SCNs was 6 to 7 min. Care of hamsters and all procedures were in full compliance with institutional guidelines for animal experimentation.
12. Polyadenylated RNA was prepared from SCN micropunches by guanidinium thiocyanate–CsCl purification followed by oligo-dT chromatography (Oligotex-dT, Qiagen). Oligo-dT–primed, double-stranded cDNA was prepared (Superscript, Gibco-BRL), and the cDNA samples were divided into two and digested with either Rsa I or Alu I, each digest being used for a separate RDA experiment. Fragments were blunt-ligated on each end to a linker containing a Bgl II site [top (A-Bgl-24), 5′-TCCAGCCTCTCACCGCAGATCTGG; bottom, 5′-CCAGATCTGCGGTGAG], and products between 150 and 1500 base pairs were gel-purified; 100 ng of the linked cDNA was used as PCR template in 4 ml of reaction mix, divided into 10 tubes (400 μl each) of PCR buffer (*10*) containing 1.25 μM A-Bgl-24 (top) primer. Samples were warmed to 72°C for 1 min, Amplitaq (Perkin-Elmer, 37.5 U/ml) was added, and 15 cycles (94°C for 1 min, 72°C for 3 min) were performed. Linkers were removed with Bgl II, and fragments from 150 to 1500 base pairs were gel-purified, generating a cDNA representation of the original mRNA to be used as tester or driver in RDA (*10*).
13. RDA was performed essentially as described (*10*), but with the following modifications. Hybridizations were for 40 hours (round 1: driver, 20 μg, tester, 200 ng; round 2: driver, 20 μg, difference product 1, 20 ng; round 3: driver, 20 μg, difference product 2, 500 fg). After round 3, linkers were removed with Bgl II, and PCR products were ligated into PCRscript (Stratagene). For forward subtractions, the cDNA representation from the sham-treated hamsters was used as the driver and that from light-treated hamsters as the tester (*10*); for reverse subtractions, the driver and tester designations were reversed.
14. Duplicate Southern blots of inserts from RDA clones were prepared. Each blot from a duplicate pair was hybridized, respectively, to ^{32}P-labeled cDNA synthesized from +light and –light cDNA representations (*12*) and then washed (twice for 20 min at 65°C, 0.1× SSC, 0.1% SDS). Filters were stripped by boiling and hybridized to subtracted probes; ^{32}P-labeled cDNA was synthesized from final RDA products from the forward and reverse subtractions (*13*), respectively, and washed as above.
15. Forty-one plasmids that were isolated in control reverse subtractions (*13*) or that corresponded to highly abundant transcripts were set aside as likely artifacts; nearly half were accounted for by three clones.
16. Syrian hamsters were entrained to a light-dark cycle (14 hours light, 10 hours dark) for ≥3 weeks, transferred to constant dim light (<1 lux) for 3 to 7 days, subjected to a light or sham treatment (*12*) at CT 19, 14, or 6, anesthetized (sodium pentobarbital, 100 mg/kg intraperitoneally), and intracardially perfused (PBS, then 4% paraformaldehyde-PBS). Brains were removed, postfixed (4% paraformaldehyde-PBS, 10% sucrose, 24 hours), frozen (methylbutane, –20°C, 2 min), and stored (–70°C). Coronal sections (16 μm, cryostat) were cut and prepared as described [D. M. Simmons, J. L. Arriza, L. W. Swanson, *J. Histotechnol.* **12**, 169 (1989)]. Sections were dried and stored (–70°C). In situ hybridization with ^{35}S-labeled riboprobes was as described [D. G. Wilkinson, J. A. Bailes, J. E. Champion, A. P. McMahon, *Development* **99**, 493 (1987)].
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18. The highest scoring matches in BLAST searches [S. F. Altschul, W. Gish, W. Miller, E. W. Myers, D. L. Lipman, *J. Mol. Biol.* **215**, 403 (1990)] for rda-7 ($P = 10^{-22}$) and rda-65 ($P = 10^{-9}$) were to different segments of the 3′ untranslated region of human *egr-3*, starting, respectively, 0.8 kb and 1.5 kb 3′ from the end of the *egr-3* coding sequence.
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Role of PML in Cell Growth and the Retinoic Acid Pathway

Zhu Gang Wang,* Laurent Delva,* Mirella Gaboli, Roberta Rivi, Marco Giorgio, Carlos Cordon-Cardo, Frank Grosveld, Pier Paolo Pandolfi†

The *PML* gene is fused to the retinoic acid receptor α (*RARα*) gene in chromosomal translocations associated with acute promyelocytic leukemia (APL). Ablation of murine PML protein by homologous recombination revealed that PML regulates hemopoietic differentiation and controls cell growth and tumorigenesis. PML function was essential for the tumor-growth–suppressive activity of retinoic acid (RA) and for its ability to induce terminal myeloid differentiation of precursor cells. PML was needed for the RA-dependent transactivation of the *p21$^{WAF1/CIP1}$* gene, which regulates cell cycle progression and cellular differentiation. These results indicate that PML is a critical component of the RA pathway and that disruption of its activity by the PML-RARα fusion protein may be important in APL pathogenesis.

Acute promyelocytic leukemia is a distinct subtype of myeloid leukemia that is invariably associated with chromosomal translocations involving the *RARα* locus (*1*). In 99% of APL cases, *RARα* is fused to the *PML* gene, leading to the production of a PML-RARα chimeric protein (*2*).

Retinoic acid receptors are nuclear hormone receptors that act as RA-inducible transcriptional activators, in their heterodimeric form, with retinoid-X receptors (RXRs), a second class of nuclear retinoid receptors (*3*). Retinoic acid controls fundamental developmental processes, induces terminal differentiation of myeloid hemopoietic progenitors, and has tumor- and cell-growth–suppressive activities (*4*). *PML* is an interferon (IFN)-inducible gene (*5*) that encodes a RING-finger protein typically concentrated within discrete speckled nuclear structures called PML nuclear bodies (PML NBs) or PML oncogenic domains (*2*, *5*). Through its ability to heterodimerize with PML and

Z. G. Wang, L. Delva, M. Gaboli, R. Rivi, M. Giorgio, P. P. Pandolfi, Department of Human Genetics and Molecular Biology Program, Memorial Sloan-Kettering Cancer Center, Sloan-Kettering Division, Graduate School of Medical Sciences, Cornell University, 1275 York Avenue, New York, NY 10021, USA.
C. Cordon-Cardo, Department of Pathology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA.
F. Grosveld, Department of Cell Biology and Genetics, Faculty of Medicine, Erasmus University, Post Office Box 1738, 3000 DR, Rotterdam, Netherlands.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: p-pandolfi@ski.mskcc.org