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The Human *MYOD1* Transgene Is Suppressed by 5-Bromodeoxyuridine in Mouse Myoblasts[1]

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5-Bromodeoxyuridine (BrdU) immediately and clearly suppresses expression of the mouse *Myod1* and human *MYOD1* genes in myoblastic cells. Despite various studies, its molecular mechanism remains unknown. We failed to identify a BrdU-responsive element of the genes in experiments in which reporter constructs containing known regulatory sequences were transferred to mouse C2C12 myoblasts. Therefore, we transferred human chromosome 11 containing the *MYOD1* gene to the cells by microcell-mediated chromosome transfer. In the resulting microcell hybrids, BrdU suppressed expression of the transgene, as determined by quantitative real-time RT-PCR analysis. We then transfected human PAC clones containing the *MYOD1* gene to the cells. In the resulting transfectants, BrdU suppressed the transgene similarly. Deletion analysis suggested that a BrdU-responsive element or chromatin structure exists between 24 and 47 kb upstream of the gene. These results are the first demonstrating BrdU-responsiveness of a transgene for the known BrdU-responsive genes and facilitating determination of its precise responsible structure.

Key words: BrdU, gene expression, *MYOD1*, PAC, transgene.

5-Bromodeoxyuridine (BrdU) is normally incorporated into DNA as a thymidine analog and widely used for various biological purposes. For instance, BrdU is well known to modulate the differentiation of cultured mammalian cells (*1–4*). These effects are based on that BrdU inhibits growth slightly and manifests its growth retardation activity slowly during culture. To date, many attempts have been made to reveal the molecular mechanism underlying the action of BrdU, but it remains a mystery. Recently, BrdU was shown to clearly induce a senescence-like phenomenon in mammalian cells regardless of the cell type or species (*5*). These finding prompted us to obtain an insight into a molecular mechanism by which BrdU specifically regulates tissue- and senescence-specific genes. We have chosen the mouse *Myod1* or human *MYOD1* gene as a model system to do so because the mouse *Myod1* gene is one of the best characterized ones in terms of the effects of BrdU.

Among the genes regulated by BrdU, the mouse *Myod1* gene is particularly interesting since its expression is immediately and clearly suppressed in mouse myoblasts. Myod1 is a member of the basic helix loop helix (bHLH) transcription factor family and acts as a major myogenic regulatory factor (MRF) of skeletal muscle differentiation, especially of determination of the skeletal muscle lineage (reviewed in Refs. *6* and *7*). Many efforts have been made to elucidate a regulatory mechanism for Myod1 expression *in vitro* and *in vivo*, but it has proved to be more complex than expected. Transcription of the *Myod1* (and *MYOD1*) gene is suppressed by histone deacetylase inhibitors (*8*), a DNA minor groove binder (*9*), cytokine (*10*), BrdU (*1*), oncogenes (*11*), RNase L (*12*), helix loop helix protein (*13*), and a HMG box binding protein (*14*). On the contrary, it is activated by MRFs (including Myod1 itself) (*15, 16*), Pax-3 (*17*), DNA methyltransferase I (*18*), the serum response factor (*19*), and 5-azacytidine (in C3H10T1/2 cells) (*20*).

In this study, first we performed conventional gene transfer experiments involving the transfer of reporter gene constructs containing known regulatory sequences of the mouse *Myod1* and human *MYOD1* genes to mouse C2C12 myoblasts, but in vain. Therefore, we transferred the human *MYOD1* gene into the cells by chromosome transfer and PAC transfection. The transgene was found to respond to BrdU for the first time as the endogenous mouse *Myod1* gene. This finding will facilitate determination of the regulatory role of BrdU in the expression of particular genes.

MATERIALS AND METHODS

Reagents—BrdU, puromycin, cytochalasin B, phytohemagglutinin, and *Taq* DNA polymerase were obtained from Sigma. DNase I and RNase inhibitor were obtained from Wako Pure Chemicals. [α-^{32}P]dCTP (3,000 Ci/mmol) was purchased from HAS. The reagents used were of reagent grade.

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Abbreviations: BrdU, 5-bromodeoxyuridine; Ct, threshold cycle; PAC, P1 phage-derived artificial chromosome; RT-PCR, reverse transcription PCR; UTR, untranslated region.

Cells and Culture Conditions—A9 (3552)-2 cells containing human chromosome 11 tagged with the neomycin resistance gene in an A9 mouse cell background (JCRB0731) (*21*) and a human embryonic rabdomyosarcoma RD cell line (JCRB9072) were obtained from the Japanese Cancer Research Resources Bank (JCRB). The mouse C2C12 myoblast cell line (*22*) was obtained from Dr. T. Endo of Chiba University. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui Seiyaku, Tokyo) supplemented with 10% fetal bovine serum (FBS; Hyclone) under a humidified atmosphere containing 5% CO_2 at 37°C. G418 (Gibco-BRL) at 800 μg/ml and 4.5 g/liter glucose were added to the medium to culture A9 (3552)-2 and C2C12 cells, respectively.

Northern Blot Analysis—Total RNA samples (10 μg) were run through 1.0% formaldehyde-agarose gels and then transferred to nylon membranes as described previously (*5, 23*). The membranes were hybridized with a probe labeled with [α-^{32}P]dCTP using a random-primed DNA-labeling kit (Mega-prime, Amersham Pharmacia Biotech) as described previously (*5*). They were washed twice at room temperature with 2× SSPE [360 mM NaCl, 20 mM sodium phosphate, and 2 mM EDTA (pH 7.7)] containing 0.1% SDS for 15 min and twice at 42 or 50°C with 1× SSPE containing 0.1% SDS for 15 min, and then exposed to an X-ray film (Fuji Photo Film).

Microcell-Mediated Chromosome Transfer—Microcell-mediated chromosome transfer was performed as described previously (*21*) with some modifications. A9 (3552)-2 cells were inoculated into 25-cm^2 flasks and then cultured for 2 days. Micronuclei were induced by treatment with colcemid (0.05 μg/ml) for 48 h in medium containing 20% FBS and 800 μg/ml G418. After filling the flasks with serum-free medium containing 10 μg/ml of cytochalasin B, enucleation was performed by centrifugation of the flasks at 10,000 ×*g* for 1 h. Microcells were collected, suspended in serum-free medium, and filtered through 8-, 5-, and 3-μm polycarbonate filters (Millipore). They were then attached to prewashed recipient cell monolayers at 37°C with 100 μg/ml of phytohemagglutinin for 15 min and fused to the cells by incubation in a 47% polyethylene glycol solution for 1 min. The cells were cultured for 1 day and then replated onto 100-mm dishes (Nunclon) containing growth medium plus 400–500 μg/ml G418. Microcell hybrids were isolated and maintained in the same medium.

PAC Transfection—PAC pDJ1082L12 (GenBank accession number AC004736) containing the human *MYOD1* gene was obtained from BACPAC Resources (Oakland, USA). PAC DNA was prepared using the standard alkaline lysis procedure (*23*). Then DNA was purified by CsCl ultracentrifugation and dissolved in TE buffer. Recipient cells were transfected with 20 μg of the circular PAC DNA and 1 μg of circular pPGKpuro (*24*) according to the standard calcium phosphate co-precipitation method (*23*). The cells were replated onto 100-mm dishes containing growth medium plus 1.0–1.5 μg/ml puromycin and then allowed to form colonies. Colonies were isolated and maintained in the same medium. Alternatively, colonies were pooled and maintained similarly.

RT-PCR Analysis—Total RNA samples were prepared from cells with an ISOGEN RNA isolation kit (Nippon Gene) in the presence of DNase I and RNase inhibitor. For conventional RT-PCR analysis, first strand cDNA was synthesized from 2 μg of each sample with an oligo $(dT)_{12-18}$ primer using a SUPERSCRIPT™ First-Strand Synthesis System for RT-PCR (Invitrogen). Aliquots of 1 μl of the samples were added to 19 μl of buffer [10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, and 0.001 μg/ml of gelatin] containing 0.2 mM each of dNTP, 1 U of *Taq* DNA polymerase, and 0.25 μM of each primer (Table I). The mixtures were subjected to PCR under the following conditions: 94°C for 2 min, 55°C for 30 s, and 72°C for 45 s for 30 cycles.

We performed quantitative real-time RT-PCR analysis with two procedures. First, RT-PCR reactions were performed using a one-step QuantiTect™ SYBR® Green RT-PCR Kit (QIAGEN) according the manufacturer's instructions. Reaction mixtures (20 μl) containing 10 μl of 2× RT-PCR master mix, 0.2 μl of RT mix, 1.0 μl of total RNA template (50 ng for mouse *vimentin* or 500 ng for human *MYOD1*), and 1.0 μl of each 5 μM primer (Table I) were incubated at 50°C for 30 min, and then subjected to PCR cycles (an initial step at 95°C for 15 min, and then 45 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s) in a Rotor-Gene™ real-time DNA amplification system (Corbett Research, Australia) according to the manufacturer's instructions. SYBR® Green fluorescence was measured after each extension step. RT-PCR products were subjected to measurement of their annealing temperatures and analysis by gel electrophoresis to verify their specificity and identity. Then a standard regression curve was drawn for each target mRNA to estimate its relative amount.

Alternatively, first-strand cDNA was synthesized with 2 μg of template and oligo $(dT)_{12-18}$ primer in a 20 μl reaction volume at 42°C for 50 min, using an Invitrogen RT-PCR system according the manufacturer's instructions. The samples were subjected to PCR in a reaction mixture (25 μl) containing 12.5 μl 2× SYBR® Green master mix (Perkin

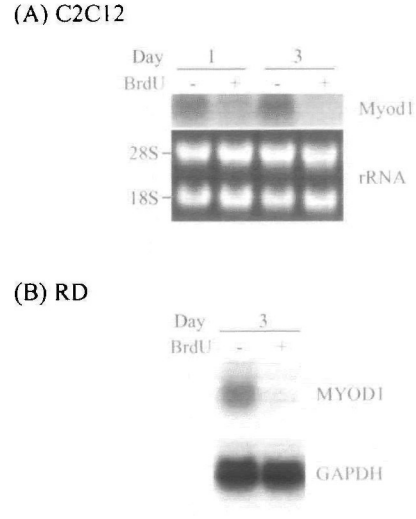

Fig. 1. **Effects of BrdU on the expression of the mouse *Myod1* and human *MYOD1* genes.** (A) Mouse C2C12 cells were cultured in the absence and presence of 10 μM BrdU for 1 and 3 days. Total RNA samples were prepared and subjected to Northern blot analysis with 1.1 kb mouse Myod1 full length cDNA as a probe as described under "MATERIALS AND METHODS." (B) Human RD cells were cultured for 3 days and processed similarly with the 0.5 kb human MYOD1 3′UTR sequence and a GAPDH sequence (*5*) as probes.

Elmer Applied Biosystems), 0.2 mM dNTP, 0.5 μl of cDNA sample, and 0.2 mM primers (Table I) under the conditions (an initial step at 95°C for 10 min, and then 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s) recommended by the manufacturer with an ABI PRISM® 7700 Sequence Detection System (Perkin Elmer Applied Biosystems), with which one can monitor fluorescent emission during PCR cycles. The RT-PCR products were analyzed by gel electrophoresis. Regression curves were drawn for each sample and its relative amount was calculated from the threshold cycles (Ct) with the instrument's software (Sequence Detector Version 1.6.3) according to the manufacturer's instructions.

RESULTS

Suppression of the Human MYOD1 Genes by BrdU—BrdU is well known to block myogenesis by preventing expression of the *Myod1* gene in mouse myoblast cell lines (*1*). We confirmed this phenomenon by Northern blot analysis in a mouse C2C12 cell line and also examined human embryonic rabdomyosarcoma cell line RD. When BrdU was added to the C2C12 cells, the level of Myod1 mRNA significantly decreased within 24 h and it became undetectable within 3 days, as reported previously (Fig. 1A). In the RD cells, the levels of MYOD1 mRNA also decreased significantly within 24 h and it also became undetectable within 3 days after the addition of BrdU (Fig. 1B and data not shown). These results demonstrate that the human *MYOD1* gene immediately responds to BrdU in human embryonic rabdomyosarcoma cells, especially since the cells divide once in 24 h and BrdU exerts its effect after incorporation into the genomic DNA.

Lack of Response to BrdU in Reporter Constructs Containing Known Regulatory Sequences—Regulatory elements controlling tissue-specific gene expression have been identified in the mouse *Myod1* and human *MYOD1* genes (*25–27*, and see Fig. 2). However, a regulatory element conferring their sensitivity to BrdU has yet to be identified. Furthermore, it is not known whether BrdU acts on such a regulatory element, if at all, in a *cis* or *trans* manner.

In initial experiments, we constructed plasmids containing mouse distal regulatory region (DRR) (*25*), human distal core enhancer (DCE) (*26, 27*), or SV40 enhancer upstream of the luciferase or green fluorescent protein (GFP) gene driven by the SV40 promoter or 2.8 kb human *MYOD1* promoter (–2.5 to +0.3 kb fragment in Fig. 2). These sequences are shown to contain the regulatory elements necessary for the human and mouse genes to be expressed normally during muscle differentiation. These plasmids were stably and transiently transfected to C2C12 cells by the calcium coprecipitation method and examined for luciferase activity or green fluorescence intensity at 1–4 days after the addition of BrdU. Unfortunately, however, we

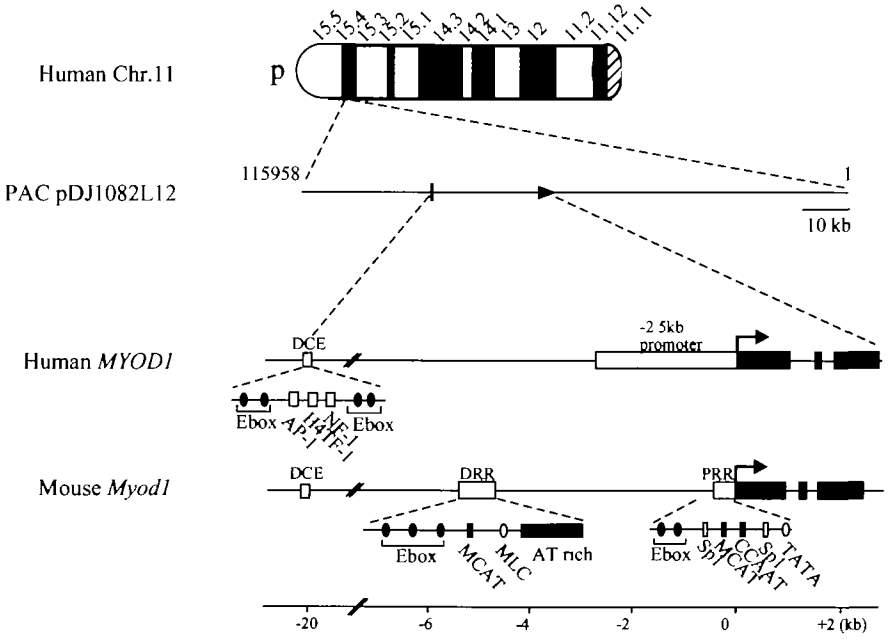

Fig. 2. **Schematic representation of the human *MYOD1* and mouse *Myod1* genes.** On BLAST and homology searches, PAC pDJ1082L12 was found to contain the whole gene and long flanking sequences on both sides (*27*). For other details, see the text.

TABLE I. **Primers used in this study.**

Primer		Sequences	Locus/gene
TH	Forward	5′-GGGTATCTGGGCTCTGGGGT-3′	Human chromosome 11pter-p15.5
	Reverse	5′-GGTCACAGGGAACACAGACTCC-3′	
–20 kb	Forward	5′-CCCGGGAGCTCCCACAGCAGTTGGGGGCATTTATG-3′	Human *MYOD1* –20 kb distal core enhancer
	Reverse	5′-CCCGGGAATTCCCCAAACCTCAGGACTCAGTTGG-3′	
3′UTR	Forward	5′-AACTTAAATGCCCCCCTCCCAAC-3′	3′ untranslated region of human *MYOD1* gene
	Reverse	5′-AGAAACGTGAACCTAGCCCCTC-3′	
Vimentin	Forward	5′-ACCTGTGAAGTGGATGCCCT-3′	3′ coding region of mouse *vimentin* gene
	Reverse	5′-AAATCCTGCTCTCCTCGCCTT-3′	

were not able to observe BrdU-responsiveness in these conventional transfection assays (data not shown).

Suppression by BrdU of the Human MYOD1 Transgene in Microcell Hybrids—We returned to intact human chromosome 11, which contains the human *MYOD1* gene at band 11p15.4 (*28*, and see Fig. 2), to search for a BrdU-responsive regulatory element. We isolated microcell hybrids of C2C12 cells by microcell-mediated transfer of human chromosome 11. The microcell hybrids isolated were subjected to genomic PCR analysis using primers for a short-arm terminal marker, TH, a sequence 20 kb upstream of the transcription start site of the *MYOD1* gene, and its 3′UTR sequence (Table I). Most of them were found to contain all of the sites, suggesting that at least the short arm of human chromosome 11 was retained (Fig. 3A). Human chromosome 11 was also detected in metaphase spreads. Then we examined the mRNA levels for the *MYOD1* gene by RT-PCR with the primers for its 3′UTR sequence (Fig. 3B). The mRNA was detected in the majority of the microcell hybrids, but the levels were lower than those for the endogenous mouse *Myod1* gene.

To quantitatively compare the mRNA levels in control and BrdU-treated cells, we performed real-time RT-PCR analysis using a Rotor-Gene™ real-time DNA amplification

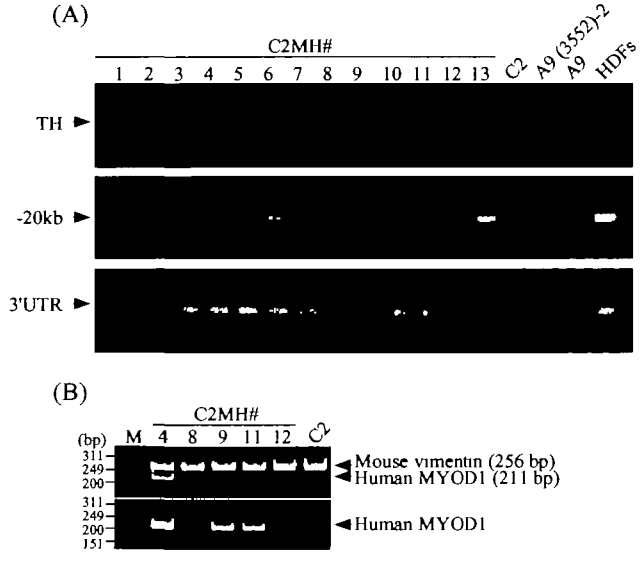

Fig. 3. **Characterization of microcell hybrids of C2C12 cells containing human chromosome 11.** (A) Genomic DNA samples were prepared from microcell hybrids with a DNA extraction kit (IsoQuick, MICROPROBE) (*5*), and then subjected to PCR with primer sets for the TH, –20 kb, and 3′UTR loci (Table I) under standard conditions. The PCR products were run on 3% agarose gels and stained with ethidium bromide. Lanes 1–13, independent microcell hybrid clones C2MH#1–13; C2, mouse C2C12 cells; A9 (3552)-2, mouse A9 cells containing human chromosome 11; A9, A9 cells; HDFs, human diploid fibroblasts. (B) Total RNA samples were prepared from microcell hybrids and then subjected to RT-PCR analysis with primer sets for human MYOD1 and mouse vimentin cDNAs (top panel) or a primer set for human MYOD1 cDNA (bottom panel) as described under "MATERIALS AND METHODS." The PCR products were run on 8% non-denaturant polyacrylamide gels and stained with ethidium bromide. Lane M, *Hin*fI digest of øX174 DNA; C2MH#4–12, a number of the microcell hybrids shown in (A).

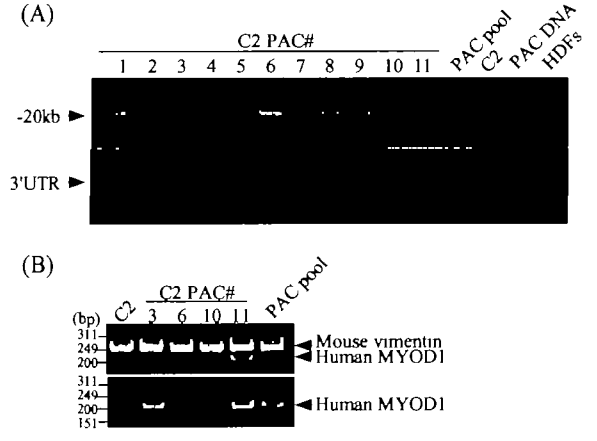

Fig. 4. **Characterization of transfectants of C2C12 cells containing human PAC DNA.** (A) Transfectant clones were subjected to genomic PCR with primer sets for the –20 kb and 3′UTR loci as in Fig. 3. Lanes 1–11, independent transfectant clones C2PAC#1–11; PAC pool, a mixture of *ca.* 300 clones; C2, mouse C2C12 cells; PAC DNA, PAC pDJ1082L12; HDFs, human diploid fibroblasts. (B) Total RNA samples were prepared from transfectants and processed as in Fig. 3. C2PAC#3–11, a number of the transfectants shown in (A).

TABLE II. **Quantitative measurement of human MYOD1 mRNA in mouse C2C12 cells containing human chromosome 11 or PAC pDJ1082L12.**[a]

Sample[b]		Human MYOD1 mRNA		Mouse vimentin mRNA		Suppression rate (1)/(2)
		Relative level[c]	Cont/BrdU (1)	Relative level	Cont/BrdU (2)	
C2MH#4	Cont	223.07	4.48	80,167.81	0.73	6.13
	BrdU	49.81		109,951.21		
C2MH#11	Cont	50.33	4.86	100,461.58	1.09	4.46
	BrdU	10.35		92,384.69		
C2PAC#6	Cont	3,285.41	16.67	185,353.16	1.75	9.53
	BrdU	197.05		105,779.13		
C2PAC#11	Cont	4,161.20	8.07	122,687.41	1.25	6.46
	BrdU	515.49		98,537.15		
C2 PAC pool	Cont	1,800.95	6.43	131,704.31	1.28	5.02
	BrdU	280.05		103,086.07		
C2C12	Cont	N.D.[d]		130,857.92	1.32	N.D.
	BrdU	N.D.		99,174.50		N.D.

[a]Real-time RT-PCR assays were performed with the Rotor-Gene™ Real-Time DNA Amplification System. [b]RNA samples were prepared from cells cultured with (BrdU) or without (Cont) 10 μM BrdU for 3 days. [c]Ct (threshold cycle) was converted to a relative expression level using the standard regression curves in Fig. 5. [d]N.D., not determined. Values represent one of two independent experiments that gave similar results.

system. The threshold cycles (Ct) at which MYOD1 mRNA accumulated to a detectable level in the samples were plotted against the dilutions of template RNA (Fig. 5). This estimation of a specific mRNA level in cells was verified by a wide range of linearity in the real-time RT-PCR analysis. In microcell hybrids, MH#4 and MH#11, chosen randomly, the expression of the human *MYOD1* gene decreased by 4–6 fold on the addition of BrdU for 3 days (Table II). Similarly, the mRNA level for the endogenous mouse *Myod1* gene decreased by approximately 3-fold in all of the above microcell hybrids, as detected on Northern blot analysis (data not shown). The amount of mouse vimentin mRNA used as a control did not differ significantly compared with that of MYOD1 mRNA (Table II).

These results showed that the human *MYOD1* transgene on human chromosome 11 clearly responds to BrdU, suggesting that a regulatory element, if any, exists on this chromosome.

Suppression by BrdU of the Human MYOD1 Transgene in PAC Transfectants—We attempted to locate a BrdU-responsive regulatory element on human PAC or BAC clones. We searched databases and found that the PAC pDJ-1082L12 clone contains the *MYOD1* gene with 47 kb of a 5′ flanking and 69 kb of a 3′ flanking sequence in its 116 kb insert (Fig. 2). We transfected this PAC clone to C2C12 cells with a plasmid encoding the puromycin resistance gene and isolated stable transfectants as to resistance to puromycin. These transfectants were subjected to genomic PCR with primers for the –20 kb and 3′UTR sequences (Fig. 4A), and then to RT-PCR with the primers for the 3′UTR sequence (Fig. 4B). The transfectants positive for the –20 kb and 3′UTR sequences expressed MYOD1 mRNA at various levels, although the levels were significantly lower than those of the endogenous mouse counterpart, as seen in the microcell hybrids.

We determined the MYOD1 mRNA levels in the C2PAC#6 and C2PAC#11 clones by real-time RT-PCR analysis. The addition of BrdU reduced their levels by 6–10-fold (Table II). The mouse vimentin mRNA levels did not change significantly. In another experiment, we pooled *ca.* 300 independent puromycin-resistant colonies, and then examined MYOD1 mRNA in the resulting mass culture. This sample showed a 5-fold reduction in the mRNA level upon the addition of BrdU (Table II). The mouse Myod1 mRNA level decreased by approximately 3-fold on the addition of BrdU in all of the above transfectants, as detected on Northern blot analysis (data not shown).

These results demonstrate that the *MYOD1* transgene on the PAC clone responds to BrdU as the endogenous mouse counterpart, suggesting the presence of a BrdU-responsive regulatory element in its vicinity.

We constructed two deletion mutants, p*Sal* and p*Not*, from PAC pDJ1082L12 (Fig. 6A). p*Sal* contains an about 51 kb DNA encompassing 47 kb of the 5′ flanking and 2 kb of the 3′ flanking sequence of the *MYOD1* gene. p*Not* contains about a 39 kb DNA encompassing 24 kb of the 5′ flanking and 13 kb of the 3′ flanking sequence. After cotransfection to C2C12 cells with the puromycin resistance

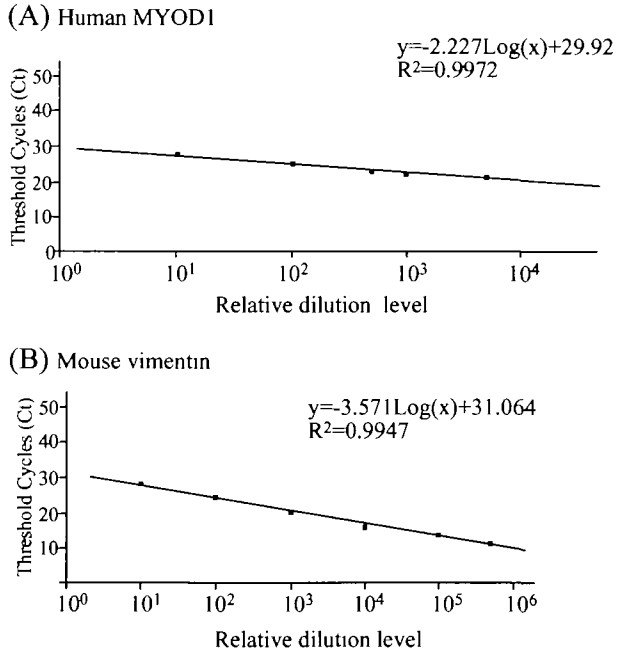

Fig. 5. **Standard regression curves for human MYOD1 and mouse vimentin mRNA species on real-time RT-PCR.** Threshold cycles (Ct) were plotted against the logarithms of successive dilutions of a template RNA sample (prepared from C2PAC#11) to obtain a linear relationship. Duplicate assays were performed for each point. The Ct values are defined as the cycle numbers at which amplification starts on RT-PCR with the primer sets for the human MYOD1 3′UTR sequence (A), and the mouse vimentin sequence (B) (Table I) in this case. According to the software's protocol, the cases in which 100 and 1 pg of the template RNA were used were defined as dilution level 1 for MYOD1 and vimentin, respectively. The dilution levels represent the relative expression levels of mRNA species. Based on a standard regression curve for mouse vimentin in each sample, the relative mRNA level for human MYOD1 was determined from its regression curve.

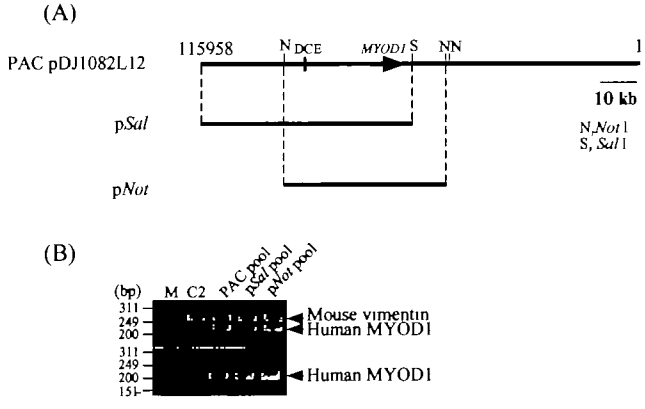

Fig. 6. **Characterization of transfectants of C2C12 cells containing a PAC deletion derivative.** (A) Schematic representation of PAC deletion derivatives. pDJ1082L12 was digested with *Sal*I, and the resulting 65 kb fragment was purified by field inversion gel electrophoresis and then self-ligated to yield PAC p*Sal*. The 39 kb *Not*I fragment was purified and cloned into the *Not*I site of the pCYPAC2 vector to yield PAC p*Not*. (B) The above deletion derivatives were transfected to C2C12 cells, and the resulting colonies were pooled and subjected to genomic PCR (not shown) and RT-PCR to detect human MYOD1 and mouse vimentin mRNA species as in Fig. 3. Lane M, *Hin*fI digest of øX174 DNA; C2, mouse C2C12 cells; PAC pool, a pool of *ca.* 300 clones transfected with PAC pDJ-1082L12; p*Sal* pool and p*Not* pool, pools of *ca.* 300 clones transfected with PAC p*Sal* and p*Not*, respectively.

TABLE III. **Quantitative measurement of human MYOD1 mRNA in mouse C2C12 cells containing a derivative of PAC pDJ1082-L12.**[a]

Sample[b]		Human MYOD1 mRNA		Mouse vimentin mRNA		Suppression rate (1)/(2)
		Relative level[c]	Cont/BrdU (1)	Relative level	Cont/BrdU (2)	
C2 PAC pool	Cont	1.12	3.11	0.90	1.43	2.18
	BrdU	0.36		0.63		
C2 p*Sal* pool	Cont	15.87	2.56	0.96	1.14	2.24
	BrdU	6.20		0.84		
C2 p*Not* pool	Cont	1.49	0.78	0.82	1.28	0.61
	BrdU	1.91		0.64		

[a]Real-time RT-PCR assays were performed with the ABI PRISM® 7700 Sequence Detection System. [b]RNA samples were prepared as in Table II. [c]Ct (threshold cycle) was converted to a relative expression level using the standard regression curves as in Fig. 5. Values represent the averages of triplicate experiments.

plasmid, *ca.* 300 independent puromycin-resistant colonies were pooled for each clone (p*Sal* pool and p*Not* pool), and the pooled cultures were found to express MYOD1 mRNA, as found on genomic PCR (data not shown) and conventional RT-PCR analysis (Fig. 6B) with the –20 kb and 3′UTR primers. Real-time RT-PCR analysis with an ABI PRISM® 7700 Sequence Detection System (Perkin Elmer Applied Biosystems) revealed that the addition of BrdU decreased the MYOD1 mRNA levels in the PAC and p*Sal* pools to significant and similar extents, but not that in the p*Not* pool (Table III). In these real-time RT-PCR analyses, the relative differences in a given mRNA level were retained although the absolute amounts differed from experiment to experiment. Despite such differences, these results suggest that a *cis*-acting regulatory element exists between –47 and –24 kb of the human *MYOD1* transgene.

DISCUSSION

To date, reporter gene constructs containing known regulatory elements of the mouse *Myod1* gene have exhibited no response to BrdU in gene transfer experiments. To overcome this, we first demonstrated that BrdU suppresses expression of the human *MYOD1* gene in RD cells, as it does that of the mouse *Myod1* gene in C2C12 cells. Then we transferred intact human chromosome 11 or PAC clones to C2C12 cells as a donor of the *MYOD1* gene. In the resulting microcell hybrids and PAC transfectants, the *MYOD1* gene was found to respond to BrdU. In both systems, however, the expression of the human *MYOD1* gene was significantly weaker than that of the endogenous mouse counterpart, probably due to a lack of a human *trans*-acting factor in C2C12 cells. Thus, it is preferable to use human cell lines such as RD instead of C2C12. Nonetheless, the above results demonstrate for the first time that BrdU suppressed a BrdU-responsive gene at the transgene level.

Deletion analysis with the PAC clone suggested the presence of a BrdU-responsive region at more than 24 kb upstream of the *MYOD1* gene. This finding is in agreement with failure to identify a BrdU-responsive *cis*-acting regulatory element in previous experiments, in which known regulatory sequences in close vicinity to the *Myod1* (*MYOD1*) gene were examined. However, it cannot be ruled out that such a regulatory element does not exist in the distal region of the gene because it is too distant from its promoter. Recently, accumulating evidence indicated that changes in higher-order chromatin structures strongly affect gene expression. Therefore, an alteration in particular chromatin structures may well be involved in the effects of BrdU. In fact, BrdU decondenses constitutive heterochromatin and A/T-rich Giemsa-dark bands in mitotic chromosomes, especially in human chromosomes 1, 9, 15, 16, and Y (*29, 30*). We recently demonstrated that substitution of thymine with 5-bromouracil in an A/T-rich scaffold/nuclear matrix attachment region (S/MAR) sequence reduces its degree of bending and increases its binding capacity as to the nuclear matrix (*31, 32*). We also performed computer analysis to search for S/MARs on the p*Sal* PAC sequence using the MAR-Wiz software (http://www.futuresoft.org/MAR-Wiz/). Consequently, two putative S/MARs were found 17 and 41 kb upstream of the human *MYOD1* gene. Furthermore, AT-minor groove binders such as distamycin A synergistically potentiate the effect of BrdU to induce senescence-associated genes located on A/T-rich Giemsa-dark bands (*32*). Distamycin A clearly inhibits expression of the *Myod1* gene in C2C12 cells (*9*). Since the human *MYOD1* gene is a typical differentiation-specific gene located on G-band 11p15.4 (*28*, and Fig. 2), it is reasonable to speculate that a change in chromatin structures is also involved in the effect of BrdU on this gene.

Finally, unfortunately, we were not able to identify the BrdU-responsive element or structure in the present study. Nonetheless, the PAC transfer system we described here will facilitate elucidation of the molecular basis for the regulatory role of BrdU in the *MYOD1* gene. This and other efforts will help to solve the old and new mysteries regarding the action of BrdU.

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