Journal of Neurochemistry
Lippincott-Raven Publishers, Philadelphia

Identification of Candidate Genes Induced by Retinoic Acid in Embryonal Carcinoma Cells

William M. W. Cheung, Apple H. Chu, and Nancy Y. Ip

Department of Biology and Biotechnology Research Institute, The Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong

Abstract: Retinoic acid (RA) induced the terminal differentiation of a human embryonal carcinoma cell line (NT2/D1) into several morphologically distinct cell types, including the postmitotic CNS neurons. Although RA has been suggested to play an important role in brain development, little is known about the molecular mechanism by which RA induces neuronal differentiation. In the present study, RNA fingerprinting by arbitrarily primed PCR (RAP-PCR) was used to identify the transcripts in NT2/D1 cells that were differentially regulated by RA. Northern blot analysis of the differentially amplified PCR fragments revealed 11 genes that were regulated by RA. Of these, seven were up-regulated and four were down-regulated along the course of RA treatment. More importantly, four of the RA-regulated genes that were identified in the present study are novel. Our findings suggested that there are a number of RA-regulated genes that have yet to be identified. RAP-PCR provides a useful tool for studying the patterns of transcript expression during the course of RA treatment and allows the cloning of novel genes involved in the process of neuronal differentiation. Furthermore, it provides a basis for the selection of genes that are involved in the RA-induced signaling pathway in the human CNS. **Key Words:** Neuronal differentiation—RNA fingerprinting by arbitrarily primed PCR—NTera2—Embryonal carcinoma—CNS differentiation.
J. Neurochem. **68,** 1882–1888 (1997).

Retinoic acid (RA), a derivative of vitamin A, induces differentiation in many cell types such as epithelial and cancer cells (De Luca, 1991). In addition to its use as therapeutic agents for the treatment of acute promyelocytic leukemia (Warrell et al., 1991), RA has been suggested to be important in CNS development when the receptors for RA and retinoids were identified in the developing nervous system (Maden et al., 1990; Dencker et al., 1991). The effects of RA are mediated by the interaction of its ligand–receptor complexes with specific DNA response elements, including the RA response elements (RARES) or retinoid X response elements (RXRES) (Leid et al., 1992). Increasing evidence has been accumulated on the role of RA in embryogenesis, such as pattern formation (Conlon, 1995; Kraft et al., 1994), but little is known on the molecular mechanism by which RA induces neuronal differentiation.

Following treatment with RA for more than 3 weeks, a human embryonal carcinoma cell line, NTera2 cl.D1 (NT2/D1), was demonstrated to differentiate into several morphologically distinct cell types, including terminally differentiated postmitotic CNS neurons (Andrews, 1984; Andrews et al., 1984; Pleasure and Lee, 1993). NT2/D1 cells represent the most extensively studied human embryonal carcinoma cells that are capable of differentiating into neurons (Andrews, 1988; Pleasure et al., 1992); these cells serve as a good model system for the study of the molecular mechanism by which RA induces neuronal differentiation in the human CNS. For example, RA was demonstrated to down-regulate the expression of growth factors, including transforming growth factor-α and basic fibroblast growth factor, and protooncogenes, such as c-*myc* and N-*myc,* while up-regulating two homeotic genes, Hox 2.1 and Hox 2.2 (Miller et al., 1990). These responses to RA were mediated through the RA nuclear receptor-γ (Moasser et al., 1994). Furthermore, a recent study from our laboratory has suggested that RA-induced CNS neuronal differentiation may be mediated through the coordinated regulation of the neurotrophin receptors (Cheung et al., 1996).

To identify stage-specific proteins, various differential cDNA screening methods have been developed. Major approaches include differential hybridization (Dworkin and David, 1980), subtractive hybridization (Travis and Sutcliffe, 1988; Watson et al., 1990),

Received November 14, 1996; revised manuscript received January 7, 1997; accepted January 7, 1997.

Address correspondence and reprint requests to Dr. N. Y. Ip at Department of Biology, The Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong.

Abbreviations used: AP-PCR, arbitrarily primed PCR; DMEM, Dulbecco's modified Eagle's medium; E-, N-, and P-cadherin, epithelial, neural, and placental cadherin, respectively; FBS, fetal bovine serum; HSPG, heparan sulfate proteoglycan; RA, retinoic acid; RAP-PCR, RNA fingerprinting by arbitrarily primed PCR; RT, reverse transcription.

PCR-based cell-specific cDNA library construction (Belyavsky et al., 1989; Welsh et al., 1990), RNA fingerprinting by arbitrarily primed PCR (RAP-PCR) (Welsh et al., 1992), and differential display (Liang and Pardee, 1992). Both RAP-PCR and differential display allow for an estimation of the expression of certain transcripts in response to a specific experimental treatment or developmental cues. In the present study, we have used RAP-PCR (Welsh et al., 1992) to evaluate the pattern of gene expression and to identify the candidate genes that are regulated during the process of RA-induced neuronal differentiation. RAP-PCR provides a basis for the selection of genes that are involved in the RA-induced differentiation in the human CNS. RNA fingerprints resulting from different patterns of transcript expression in response to RA treatment of NT2/D1 cells were compared. Differentially amplified products were cloned and sequenced, and their expression during the course of RA treatment in NT2/D1 cells was examined by northern blot analysis.

MATERIALS AND METHODS

Cell culture

NT2/D1 cells were cultured as previously described (Pleasure et al., 1992). In brief, cells were seeded at 2×10^6 cells per 75-cm^2 flask in Dulbecco's modified Eagle's medium (DMEM; high glucose formulation; GIBCO) supplemented with 10% fetal bovine serum (FBS; GIBCO) and were subsequently maintained in OptiMEMI reduced-serum medium (GIBCO) supplemented with 5% FBS. Cells were differentiated with 10 μM all-*trans* RA (Sigma) in DMEM supplemented with 10% FBS. Fresh medium was replenished every 3 days for various intervals (0–14 days).

RNA preparation and northern analysis

Total RNA was prepared using TRIZOL reagent (GIBCO). Ten micrograms of total RNA prepared from cells treated with RA for various intervals was electrophoresed through a formaldehyde agarose gel and transferred onto a nylon filter (MSI). Adult rat brain or liver RNAs were included as controls in each gel. Equal loading of RNA samples was indicated by the similar intensity of the ribosomal RNA bands on the ethidium bromide-stained gel. Hybridization was performed using a random-^{32}P-labeled purified cDNA probe at 60°C in phosphate buffer. Membranes were exposed to Kodak X-Omat film at −70°C for 1–7 days using Lightning Plus intensifying screens.

Arbitrary primers

Primers used in RAP-PCR included HNest5 primer (5′-GCGGTACCTCACCCTTGCCTGCTACCC; downstream primer for amplifying the human nestin gene), HTrkB5A primer (5′-GGATCGATGGCTGCTAGGGATGTCGTC; downstream primer for amplifying the human neurotrophin receptor *trkB*), HVim5 primer (5′-GGGGTACCGCTTCGCCAACTACATCG; human vimentin downstream primer), M13seq primer (5′-GTAAAACGACGGCCAGT; M13 [−20] sequencing primer), and M13rev primer (5′-GGAAACAGCTATGACCATG; M13 reverse sequencing primer). HNest5 and HVim5 primers were used for the reverse transcription (RT) reactions, whereas HTrkB5A, M13seq, and M13rev primers were used for arbitrarily primed PCR (AP-PCR).

RAP-PCR

RAP-PCR was performed as described (Welsh et al., 1992). In brief, 10 μl of each RNase-free DNaseI (Promega)-treated total RNA (5 and 15 ng/μl) was combined with 10 μl of 2× RT mix, which contained 2× Superscript first-strand buffer, 20 mM dithiothreitol, 200 μM each deoxynucleotide triphosphate, 20 U of RNasin (Promega), 2 μM primer, and 200 U of Superscript II RNaseH$^-$ reverse transcriptase. RT was performed at 37°C for 1 h. Twenty microliters of AP-PCR master mix, which contains 2× *Taq* polymerase buffer, 4 mM $MgCl_2$, 200 μM each deoxynucleotide triphosphate, 1 μM primer, 0.1 U of *Taq* polymerase (GIBCO), and 0.4 μl of [γ-^{32}P]dCTP. AP-PCR was composed of a low-stringency cycle followed by 30 high-stringency cycles. During the low-stringency cycle, the reaction mix was allowed to go through a denaturing step at 94°C for 5 min, annealed at 35°C for 5 min, and then extended for 5 min at 72°C. High-stringency cycles include denaturation at 94°C for 1 min, annealing at 55°C for 1.5 min, and extension for 1.5 min at 72°C. Four microliters of amplified products was mixed with 18 μl of 95% formamide and heated at 94°C for 2 min, and 2.5 μl of this heated mixture was electrophoresed in denaturing 6% polyacrylamide gel. Differentially expressed PCR product bands were excised from the gel and reamplified with the primers for RT and AP-PCR for 30 cycles in the absence of radioactive isotopes. Reamplified cDNA was gel-purified with Qiaex (Qiagen) and cloned.

Cloning and sequencing analysis

Purified cDNA fragments were cloned using the pCRScript SK+ cloning kit (Stratagene) as described by the vendor. Plasmid DNAs were prepared by the alkaline lysis method, and double-stranded DNA sequencing was performed with the T3/T7 primer set using the T7 DNA polymerase sequencing kit (Pharmacia). The average length of the clones obtained was ~450 bp. The rate of false positives, including the possibilities of isolation of rRNA genes, was ~20%.

RESULTS

RAP-PCR

To identify transcriptionally regulated genes that are potentially involved in the RA-induced neuronal differentiation process, RAP-PCR was performed using total RNA derived from NT2/D1 cells treated with or without RA for various intervals. In the isolation of early-response genes, RNA fingerprinting patterns for no RA treatment versus treatment for 1, 2, and 3 days were compared, whereas patterns obtained for RA treatment of 3 or 7 days were used to identify late-response genes. To compensate for the possible occurrence of differentially amplified products that reflect the concentration or quality difference among the RNA samples (McClelland et al., 1994), each RNA sample was fingerprinted in at least two threefold dilutions. Only differentially amplified products reproducibly present in both dilutions were considered as positively selected bands. RAP-PCR amplifications with five dif-

TABLE 1. *RA-regulated genes isolated by RAP-PCR*

Clone	RT primer	AP-PCR primer	Expression pattern	Gene identity	Homology
1.31	HNest5	HTrkB5A	Up-regulated	Novel	
7.31	HNest5	HTrkB5A	Constitutively expressed	Human clathrin heavy chain	100%
8.31	HNest5	HTrkB5A	Down-regulated	Novel	
12.33	HNest5	HTrkB5A	Up-regulated	Human CD59	100%
15.31	HNest5	HTrkB5A	Down-regulated	Human dystroglycan	82%
19.41	HNest5	HTrkB5A	Constitutively expressed	Novel	
28.19	HVim5	M13seq	Up-regulated	Human transcription factor SUPT4H	100%
29.2	HVim5	M13seq	Up-regulated	Novel	
30.2	HVim5	M13rev	Up-regulated	Human HSPG	100%
32.2	HVim5	M13seq	Up-regulated	Human cadherin-6	95%
37.6	M13seq	M13rev	Down-regulated	DEAD-box protein	100%
41.9	M13seq	M13rev	Down-regulated	Monocarboxylate transporter	92%
44.4	M13seq	M13rev	Up-regulated	Novel	

The RT primer and the AP-PCR primer were used for the first-strand synthesis and AP-PCR in RAP-PCR, respectively. The expression pattern was obtained by northern analysis using total RNA prepared from NT2/D1 cells treated with RA for various intervals. Gene identity indicates the gene producing the highest scoring segment pair when the cloned amplified RAP-PCR product was aligned to the deduced amino acid sequences using databases listed in the text. Homology indicates the percentage of homology between the deduced amino acid sequence of the cloned amplified RAP-PCR product and the gene identity listed.

ferent combinations of the primers (as described in Materials and Methods) revealed 11 differentially expressed patterns. Two constitutively expressed bands were selected to serve as controls in subsequent analysis.

Sequence analysis of the cloned differentially expressed PCR fragments

Differentially amplified products were reamplified with the arbitrary primers that were used for RT and AP-PCR. The reamplified products were cloned into pCRScript SK+ vector and were then sequenced using T3 and T7 primers. In most cases, more than three individual clones were being sequenced for each amplified product. The nucleotide sequences were compared with the GenBank, EMBL, DDBJ, and PDB databases. Deduced amino acid sequences were compared with the CDS translations of the GenBank database and the amino acid sequences from the PDB, SwissProt, Spupdate, and PIR databases. In both cases, the BLAST server provided by the National Center for Biotechnology Information (National Library of Medicine, National Institutes of Health) was used for performing the sequence analysis. TBLASTX was also attempted with the dbEST database at the National Center for Biotechnology Information. Clones obtained by RAP-PCR can be classified into three groups: constitutively expressed or up-regulated or down-regulated during the course of RA treatment (Table 1). Among the 13 amplified bands, five novel genes were obtained. Three of these novel clones were up-regulated, whereas one was down-regulated during RA treatment. Furthermore, one of the two constitutively expressed control bands isolated was found to be novel.

Clones 1.31, 7.31, 8.31, 12.33, 15.31, and 19.41 were isolated using HNest5 primer as an arbitrary primer for RT and HTrkB5A primer as an arbitrary primer for AP-PCR. Using HVim5 as the arbitrary primer for RT, M13seq primer yielded clones 28.19, 29.2, and 32.2, whereas M13rev primer gave clone 30.2 when used for AP-PCR. Similarly, the combination of M13seq as RT primer and M13rev as AP-PCR primer yielded clones 37.6, 41.9, and 44.4 (Table 1).

RNA blot analysis of RA-induced differentially expressed cDNA fragments

The cloned amplified products were analyzed by northern blot analysis to examine their pattern of expression in NT2/D1 cells during the course of RA treatment (3–14 days).

Seven clones, including 12.33, 28.19, 30.2, 32.2, 1.31, 29.2, and 44.4, were found to be up-regulated during RA treatment of NT2/D1 cells. Clone 12.33, which shares 100% homology with the nucleotide sequence of human CD59, an LY-6-like protein expressed in human lymphoid cells (Davies et al., 1989), and clone 30.2, which shares 100% nucleotide sequence homology with the 3′ end of the core protein of human heparan sulfate proteoglycan (HSPG), were transcriptionally induced after 7 and 14 days of RA treatment (Fig. 1A and C). A prominent transcript (~6 kb) for clone 28.19 was detected following 3 days of RA treatment; this clone was found to be 100% homologous to the human transcription factor SUPT4H mRNA (Fig. 1B). Clone 32.2 shares 95% homology with mRNA encoding the human cadherin-6 gene; three RA-induced transcripts were detected at day 7, and the expression was reduced at day 14 (Fig. 1D).

The expression of three novel clones, 1.31, 29.2, and 44.4, was up-regulated during the course of RA treatment. Basal level of expression for all three novel genes could be detected when the NT2/D1 cells were cultured in the absence of RA. Although basal expression of the transcript for clone 1.31 was low, it was

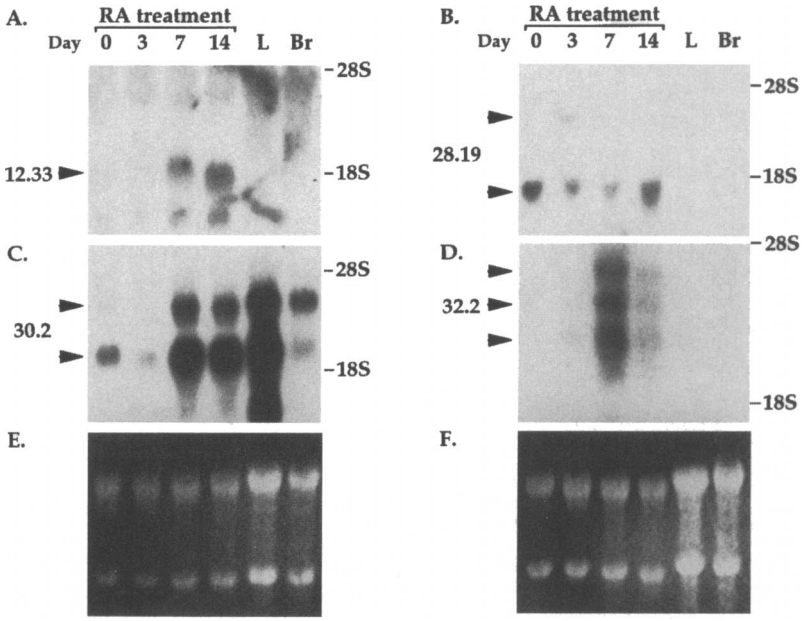

FIG. 1. Northern blot analysis of transcripts encoded by RAP-PCR clones that were up-regulated following RA treatment. NT2/D1 cells were treated with RA (10 μM) for 0, 3, 7, or 14 days. Total RNA was prepared, and northern blot analysis was performed using differentially amplified RAP-PCR fragments as cDNA probes for hybridization. Clone 12.33 encodes CD59 (**A**), clone 28.19 encodes the human transcription factor *SUPT4H* gene (**B**), clone 30.2 encodes the core protein of human HSPG (**C**), and clone 32.2 encodes the human cadherin gene (**D**). L, liver; Br, brain. **E** and **F:** Ethidium bromide-stained gel is shown to indicate that equal amounts of RNA were loaded (10 μg per sample). Positions of the ribosomal RNA bands 28S and 18S are as indicated.

induced and peaked at day 7. Clone 1.31 did not cross-hybridize with the rat brain and liver control (Fig. 2A), which might be due to the low sequence homology of this gene with that of its rat counterparts. Alternatively, it is also possible that the partially cloned novel gene was not expressed in both the adult brain and liver. The expression of clone 44.4 remained constant at day 0 and day 3 but was induced at day 7 and remained constant at day 14. Similar to clone 1.31, no cross-hybridization was detected in rat brain or rat liver for clone 44.4 (Fig. 2B). The expression of the novel clone 29.2 was induced steadily along the course of RA treatment. It is interesting that the transcript for clone 29.2 was detected in rat brain but not liver (Fig. 2C).

RNA blot analysis of RA-down-regulated differentially expressed cDNA fragments

Among the differentially amplified clones, clones 8.31, 15.31, 37.6, and 41.9 were demonstrated to be down-regulated along the course of RA treatment. Clone 8.31 shows no homology with any published DNA sequences or amino acid sequences in the databases. Northern blot analysis of this novel sequence

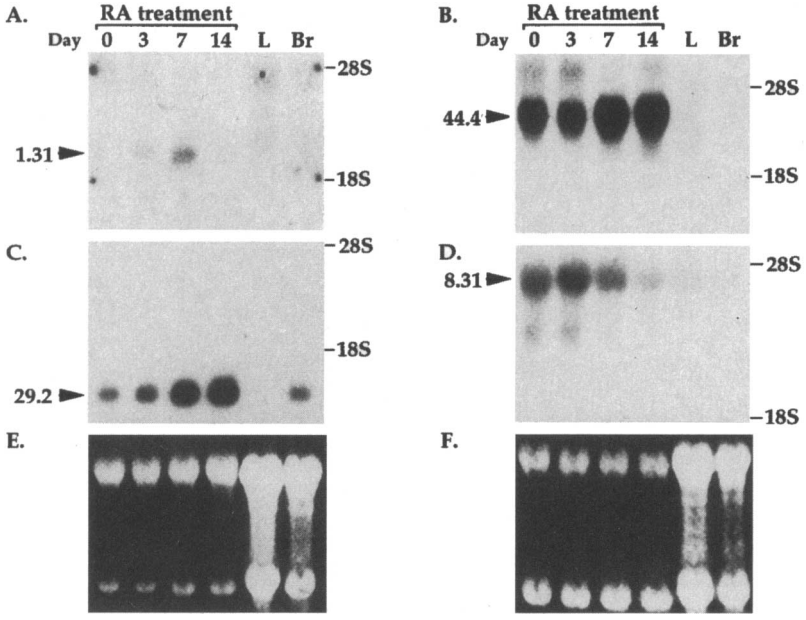

FIG. 2. Northern blot analysis of transcripts encoded by RAP-PCR clones containing novel genes that were differentially expressed in NT2/D1 cells following RA treatment. Expression of transcripts encoded by clone 1.31 (**A**), clone 44.4 (**B**), and clone 29.2 (**C**) was up-regulated in NT2/D1 cells along the course of RA treatment. Expression of mRNA for transcripts encoded by clone 8.31 was down-regulated (**D**). L, liver; Br, brain. **E** and **F:** Ethidium bromide-stained gel is shown to indicate that equal amounts of RNA were loaded (10 μg). Ribosomal RNA bands (18S and 28S) are indicated on the right.

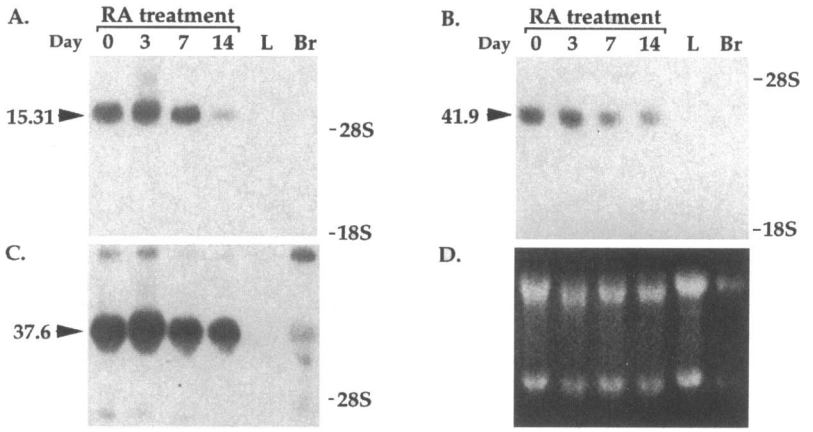

FIG. 3. Northern blot analysis of transcripts encoded by RAP-PCR clones that were down-regulated in NT2/D1 cells following RA treatment. Clone 15.31 encodes human dystroglycan (**A**), clone 41.9 encodes monocarboxylate transporter (**B**), and clone 37.6 encodes DEAD-box protein (**C**). L, liver; Br, brain. **D:** Ethidium bromide gel is shown to indicate that equal amounts of RNA were loaded (10 μg). Ribosomal bands (18S and 28S) are indicated on the right.

revealed a ~5-kb transcript that was down-regulated from day 0 to day 7 following RA treatment. However, similar to clones 1.31 and 44.4, no signal for clone 8.31 was detected in rat brain or liver (Fig. 2D). The RA-induced down-regulation was also observed for clone 15.31 (Fig. 3A); this clone shares 82% homology with the amino acid sequence of human dystroglycan. Similarly, another differentially amplified gene (clone 41.9), showing 92% homology with the mouse monocarboxylate transporter gene, was slightly down-regulated along the course of RA treatment (Fig. 3B). The expression of clone 37.6, which is 100% homologous to the deduced amino acid sequence of the DEAD-box protein, was decreased at 7 days after RA treatment (Fig. 3C).

Clathrin gene (clone 7.31), isolated as a band constitutively amplified during RA treatment, was constitutively expressed along the course of RA treatment studied (Fig. 4A). It is interesting that another clone, 19.41, that showed constitutive expression from day 0 to day 14 revealed no homology with any known sequences. The expression of its transcript was found to be at least three times higher in rat brain than in rat liver (Fig. 4B).

DISCUSSION

Our studies were aimed at the identification of genes that are differentially regulated in response to RA treatment. This provides clues for the studies of the genes that are involved in RA-induced neuronal differentiation in the human CNS. With the use of five pairs of arbitrarily chosen primers, which have been designed for use in other studies, 11 differentially expressed bands were isolated. Among these differentially amplified PCR fragments, seven of them were demonstrated to be up-regulated, whereas four of them were down-regulated during RA treatment. Two constitutively expressed bands were isolated as controls for the present study. Among the 13 clones thus far isolated and examined, five of them were novel clones, sharing no homology with any nucleic acid or amino acid sequences available in the databases listed.

Human transcription factor SUPT4H was found to share 42% homology with the *Spt4p* gene of *Saccharomyces cerevisiae.* Spt4p is crucial for normal chromatin structure and transcription in *S. cerevisiae,* but SUPT4H has only been recently demonstrated to encode a nuclear protein that is expressed in all human tissues examined (Hartzog et al., 1996). Little has been discussed on the role of this transcription factor in the neuronal differentiation process. Another gene of interest is the HSPG gene, encoded by clone 30.2. Different HSPGs have been demonstrated to be important in embryogenesis and brain development (Gould et al., 1995; Watanabe et al., 1995). HSPGs

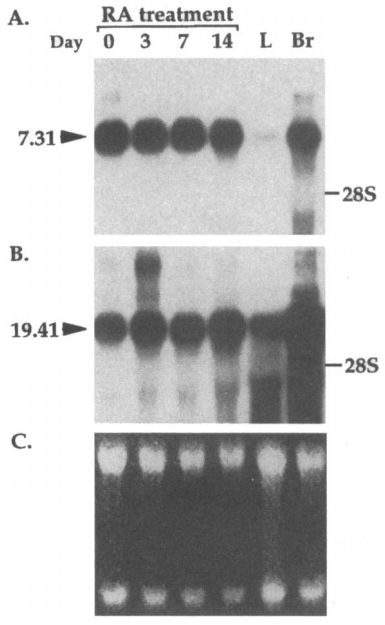

FIG. 4. Northern blot analysis of transcripts encoded by RAP-PCR clones that were constitutively expressed in NT2/D1 cells during RA treatment. Clone 7.31 (**A**) encodes the clathrin gene, and clone 19.41 (**B**) encodes a novel gene expressed constitutively in all RA-treated samples (day 0–14). L, liver; Br, brain. **C:** Ethidium bromide stained gel is shown to indicate that equal amounts of RNA were loaded (10 μg).

have also been suggested to play important roles in regulating the responses of neuronal precursor cells to particular growth factors (Ford et al., 1994). The level of a specific HSPG, perlecan, was previously demonstrated to be markedly increased during neuronal differentiation in a murine embryonal carcinoma cell line, P19. This increase was demonstrated to be concomitant with an increase in the transcriptional expression of β-amyloid protein (Sekiguchi et al., 1994). Studies are in progress in our laboratory to examine the functional role of RA-induced HSPG in CNS neuronal differentiation.

Clone 32.2 encodes the human cadherin-6 gene (Shimoyama et al., 1995), which was recently isolated as another cadherin molecule that is expressed in human cancer cells besides epithelial (E-) and placental (P-) cadherins. Previous studies have demonstrated that various combinations of the three cadherins, E-, P-, and neural (N-) cadherins, were present in different stages of NT2/D1 cells. Similar levels of P-cadherin and E-cadherin were detected in untreated NT2/D1 cells and nonneuronal subpopulations of RA-differentiated NT2/D1 cells but not detected in the neuronal population of differentiated NT2/D1 cells. In contrast, a low level of N-cadherin was detected in undifferentiated NT2/D1 cells, whereas the highest level of N-cadherin was detected in NT2/D1 neurons (Ackerman et al., 1994).

The dystroglycan gene (clone 15.31) was downregulated in NT2/D1 cells during RA treatment. Two forms of dystroglycan (α and β) encoded by the same dystroglycan gene are components of the dystrophin-associated membrane glycoprotein complex. This dystrophin–dystroglycan complex is important in myogenesis and is proposed to be a link between the extracellular matrix and the cytoskeleton. The expression of dystroglycan is ubiquitous throughout development, especially in cardiac, skeletal, and smooth muscle. Their presence in the ependymal cell lining of the developing neural tube and brain has also been reported (Schofield et al., 1995). The expression of DEAD-box protein (clone 37.6) was demonstrated to be downregulated following RA treatment in the present study. One of the DEAD-box proteins, Vasa, has recently been found to be essential in the germ line development of *Drosophila* (Liang et al., 1994). This finding raises the possibility that the high expression level of the DEAD-box protein in NT2/D1 cells might represent yet another embryonal carcinoma marker as in the case of SSEA-1 (Andrews, 1984). The decrease in the expression of DEAD-box protein concomitant with the differentiation of NT2/D1 cells might indicate the deviation of the cells from its embryonic phenotype. It is interesting that DEAD-box protein has recently been suggested to play an important role in the control of cell growth and division (Rafti et al., 1996).

The differentially amplified bands can be classified in accordance with their responses to RA treatment or, alternatively, based on their roles involved in neuronal differentiation. Among the cloned differentially amplified RAP-PCR products that are identified in the present study, there are indeed examples that can potentially represent different stages of the process of neuronal differentiation. For example, there is the transcription factor SUPT4H, which is crucial for transcription and normal formation of chromatin, the RNA-binding proteins encoded by the DEAD-box genes, which are essential for translation and might also be involved in regulating the cell growth and division, cadherin, which may be involved in the axonal outgrowth and sorting process, and dystroglycan, which is implicated in neuronal connectivities. More importantly, we have identified a novel gene (clone 29.2) that is predominantly expressed in brain tissues (authors' unpublished data).

RAP-PCR can provide insight on the identity of candidate genes that are being regulated and on the selection of potential genes for further analysis. The isolation of several novel clones in the present study suggested that there are still several RA-responsive novel genes yet to be identified. By comparing isogenic cells subjected to different experimental treatment or with different developmental status, RAP-PCR reveals the difference in the pattern of gene expression. As demonstrated in the present study, RAP-PCR provides a tool for the identification of novel genes that are involved in different physiological processes (Welsh et al., 1992). Furthermore, RAP-PCR can be used to investigate how the population of transcripts, i.e., regulation of mRNA expression, is being influenced without cloning a gene. Responding genes can be categorized and aligned to the respective treatment applied (McClelland et al., 1994). Similarly, differential display has been used to investigate the genes differentially expressed in prenatal and neonatal mammalian brain (Dalal et al., 1994; Joseph et al., 1994). Further studies are in progress in our laboratory to characterize the novel genes identified in the present study, to examine whether they are specific to the neuronal pathway, and to investigate how these novel genes are related to the RA-induced neuronal differentiation process.

Acknowledgment: We thank Dr. H. S. Kwan at the Chinese University of Hong Kong for his enthusiastic support and helpful discussion. This study was supported by the Research Grants Council of Hong Kong (HKUST 529/94M) and the Biotechnology Research Institute of HKUST.

REFERENCES

Ackerman S. L., Knowles B. B., and Andrews P. W. (1994) Gene regulation during neuronal and non-neuronal differentiation of NTERA2 human teratocarcinoma-derived stem cells. *Mol. Brain Res.* **25,** 157–162.

Andrews P. W. (1984) Retinoic acid induces neuronal differentiation of a cloned human embryonal carcinoma cell line *in vitro*. *Dev. Biol.* **103,** 285–293.

Andrews P. W. (1988) Human teratocarcinomas. *Biochim. Biophys. Acta* **948,** 17–36.

Andrews P. W., Damjanov I., Simon D., Banting G. S., Carlin C.,

Dracopoli N. C., and Fogh J. (1984) Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line Tera-2. *Lab. Invest.* **50,** 147–162.

Belyavsky A., Vinogradova T., and Rajewsky K. (1989) PCR-based cDNA library construction: general cDNA libraries at the level of a few cells. *Nucleic Acids Res.* **17,** 2919–2932.

Callard D., Lescure B., and Mazzolini L. (1994) A method for the elimination of false positives generated by the mRNA differential display technique. *Biotechniques* **16,** 1096–1103.

Cheung W. M. W., Chu A. H., Leung M.-F., and Ip N. Y. (1996) Induction of trk receptors by retinoic acid in a human embryonal carcinoma cell line. *Neuroreport* **7,** 1204–1208.

Conlon R. A. (1995) Retinoic acid and pattern formation in vertebrates. *Trends Genet.* **11,** 314–319.

Dalal S. S., Welsh J., Tkachenko A., Ralph D., DiCicco-Bloom E., Bordas L., McClelland M., and Chada K. (1994) Rapid isolation of tissue-specific and developmentally regulated brain cDNAs using RNA arbitrarily primed PCR (RAP-PCR). *J. Mol. Neurosci.* **5,** 93–104.

Davies A., Simmons D. L., Hale G., Harrison R. A., Tighe H., Lachmann P. J., and Waldmann H. (1989) CD59, an LY-like protein expressed in human lymphoid cells, regulates the action of the complement membrane attack complex on homologous cells. *J. Exp. Med.* **170,** 637–654.

De Luca L. M. (1991) Retinoids and their receptors in differentiation, embryogenesis, and neoplasia. *FASEB J.* **5,** 2924–2933.

Dencker L., Gustafson A. L., Annerwall E., Busch C., and Eriksson U. (1991) Retinoid-binding proteins in craniofacial development. *J. Craniofac. Genet. Dev. Biol.* **11,** 303–314.

Dworkin M. B., and David I. B. (1980) Use of a cloned library for the study of abundant poly(A)$^+$ RNA during *Xenopus laevis* development. *Dev. Biol.* **76,** 449–464.

Ford M. D., Bartlett P. F., and Nurcombe V. (1994) Co-localization of FGF-2 and a novel heparan sulphate proteglycan in embryonic mouse brain. *Neuroreport* **5,** 565–568.

Gould S. E., Upholt W. B., and Kosher R. A. (1995) Characterization of chicken syndecan-3 as a heparan sulfate proteoglycan and its expression during embryogenesis. *Dev. Biol.* **168,** 438–451.

Hartzog G. A., Basrai M. A., Ricupero-Hovasse S. L., Hieter P., and Winston F. (1996) Identification and analysis of a functional human homolog of the SPT4 gene of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **16,** 2848–2856.

Joseph R., Dou D., and Tsang W. (1994) Molecular cloning of a novel mRNA (neuronatin) that is highly expressed in neonatal mammalian brain. *Biochem. Biophys. Res. Commun.* **201,** 1227–1234.

Kraft J. C., Schuh T., Juchau M., and Kimelman D. (1994) The retinoid X receptor ligand, 9-*cis*-retinoic acid, is a potential regulator of early *Xenopus* development. *Proc. Natl. Acad. Sci. USA* **91,** 3067–3071.

Leid M., Kastner P., and Chambon P. (1992) Multiplicity generates diversity in the retinoic acid signaling pathways. *Trends Biochem. Sci.* **17,** 427–433.

Liang L., Diehl-Jones W., and Lasko P. (1994) Localization of vasa protein to the *Drosophila* pole plasm is independent of its RNA-binding and helicase activities. *Development* **120,** 1201–1211.

Liang P. and Pardee A. (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257,** 967–971.

Maden M., Ong D. E., and Chytil F. (1990) Retinoid-binding protein distribution in the developing mammalian nervous system. *Development* **109,** 75–80.

McClelland M., Ralph D., Cheng R., and Welsh J. (1994) Interactions among regulators of RNA abundance characterized using RNA fingerprinting by arbitrarily primed PCR. *Nucleic Acids Res.* **22,** 4419–4431.

Miller W. H. Jr., Moy D., Li A., Grippo J. F., and Dmitrovsky E. (1990) Retinoic acid induces down-regulation of several growth factors and proto-oncogenes in a human embryonal cancer cell line. *Oncogene* **5,** 511–517.

Moasser M. M., DeBlasio A., and Dmitrovsky E. (1994) Response and resistance to retinoic acid are mediated through the retinoic acid nuclear receptor γ in human teratocarcinoma. *Oncogene* **9,** 833–840.

Pleasure S. J. and Lee V. M.-Y. (1993) NTera 2 cells: a human cell line which displays characteristics expected of a human committed neuronal progenitor cell. *J. Neurosci. Res.* **35,** 585–602.

Pleasure S. J., Page C., and Lee V. M.-Y. (1992) Pure, postmitotic, polarized human neurons derived from NTera 2 cells provide a system for expressing exogenous proteins in terminally differentiated neurons. *J. Neurosci.* **12,** 1802–1815.

Rafti F., Scarvelis D., and Lasko P. F. (1996) A *Drosophila melanogaster* homologue of the human DEAD-box gene DDX1. *Gene* **171,** 225–229.

Schofield J. N., Gorecki D. C., Blake D. J., Davies K., and Edwards Y. H. (1995) Dystroglycan mRNA expression during normal and mdx mouse embryogenesis: a comparison with utrophin and the apo-dystrophins. *Dev. Dyn.* **204,** 178–185.

Sekiguchi R. T., Potter-Perigo S., Braun K., Miller J., Ngo C., Fukuchi K., Wight T. N., Kimata K., and Snow A. D. (1994) Characterization of proteoglycans synthesized by murine embryonal carcinoma cells (P19) reveals increased expression of perlecan (heparan sulfate proteoglycan) during neuronal differentiation. *J. Neurosci. Res.* **38,** 670–686.

Shimoyama Y., Gotoh M., Terasaki T., Kitajima M., and Hirohashi S. (1995) Isolation and sequence analysis of human cadherin-6 complementary DNA for the full coding sequence and its expression in human carcinoma cells. *Cancer Res.* **55,** 2206–2211.

Travis G. H. and Sutcliffe J. G. (1988) Phenol emulsion-enhanced DNA-driven subtractive cDNA cloning: isolation of low-abundance monkey cortex-specific mRNAs. *Proc. Natl. Acad. Sci. USA* **85,** 1696–1700.

Warrell R. P. Jr., Frankel S. R., Miller W. H. Jr., Scheinberg D. A., Itri L. M., Hittelman W. N., Vyas R., Andreeff M., Tafuri A., Jakubowski A., Gabrilove J., Gordon M. S., and Dmitrovsky E. (1991) Differentiation therapy of acute promyelocytic leukemia with tretinoin (all-*trans*-retinoic acid). *N. Engl. J. Med.* **324,** 1385–1393.

Watanabe K., Yamada H., and Yamaguchi Y. (1995) K-glypican: a novel GPI-anchored heparan sulfate proteoglycan that is highly expressed in developing brain and kidney. *J. Cell Biol.* **130,** 1207–1218.

Watson J. B., Battenberg E. F., Wong K. K., Bloom F. E., and Sutcliffe J. G. (1990) Subtractive cDNA cloning of RC3, a rodent cortex-enriched mRNA encoding a novel 78 residue protein. *J. Neurosci. Res.* **26,** 397–408.

Welsh J., Liu J.-P., and Efstratiadis A. (1990) Cloning of PCR-amplified total cDNA: construction of a mouse oocyte cDNA library. *Genet. Anal. Tech. Appl.* **7,** 5–17.

Welsh J., Chada K., Dalal S. S., Ralph D., Cheng R., and McClelland M. (1992) Arbitrarily primed PCR fingerprinting of RNA. *Nucleic Acids Res.* **20,** 4965–4970.