Biochem. J. (1991) 278, 871–874 (Printed in Great Britain)

Levels of ornithine decarboxylase genomic sequences, heterogeneous nuclear RNA and mRNA in human myeloma cells resistant to α-difluoromethylornithine

Juha-Matti HYTTINEN, Maria HALMEKYTÖ, Leena ALHONEN and Juhani JÄNNE*
Department of Biochemistry and Biotechnology, University of Kuopio, P.O. Box 1627, SF-70211 Kuopio, Finland

We previously isolated and characterized a human myeloma cell line overproducing ornithine decarboxylase (ODC) due to gene amplification [Leinonen, Alhonen-Hongisto, Laine, Jänne & Jänne (1987) Biochem. J. **242**, 199–203]. We have now employed the PCR combined with reverse transcription to determine semiquantitatively ODC gene dosage and the amounts of heterogeneous nuclear (hn) RNA and of mature mRNA of the enzyme in parental and α-difluoromethylornithine-resistant human myeloma cells. Experiments with dilution series revealed that the ODC gene copy number and the amount of both hnRNA and mRNA were increased to the same extent (about 100-fold) in the resistant cells. Similar dot-blot analyses of ODC-specific genomic DNA and total RNA indicated that the ODC gene copy number was increased by a factor of 380 and the amount of ODC mRNA by a factor of 700. Our results indicate that the PCR combined with reverse transcription is at least as useful as blot analyses to give semiquantitative assessment of the amounts of specific DNA or RNA sequences. In addition, the use of the PCR enables the analysis of minute sample amounts in extremely short time.

INTRODUCTION

The human genome contains two ornithine decarboxylase (ODC)-encoding loci: an actively expressed and amplifiable gene on the short arm of chromosome 2 (Winqvist *et al.*, 1986) and a processed pseudogene on chromosome 7 (Winqvist *et al.*, 1986; Hickok *et al.*, 1990). Human tumour cells acquire resistance to α-difluoromethylornithine (DFMO), a mechanism-based irreversible inhibitor of ODC (Metcalf *et al.*, 1978), by at least two entirely different mechanisms: (i) by an overproduction of ODC due to gene amplification on chromosome 2 (Leinonen *et al.*, 1987) or (ii) through an induction and/or overproduction of arginase (Alhonen-Hongisto *et al.*, 1987), resulting in an enhanced intracellular accumulation of ornithine apparently competing with DFMO (Hirvonen *et al.*, 1989). In contrast with some mouse tumour-cell lines in which the development of resistance may be associated with enhanced transcriptional activity at normal gene dosage (Alhonen-Hongisto *et al.*, 1985; McConlogue *et al.*, 1986) or in the presence of moderate gene amplification (Kahana & Nathans, 1984), human tumour cells appear to overproduce ODC roughly to the extent that could be predicted from the elevated gene copy number (Leinonen *et al.*, 1987).

In the present paper we have attempted to quantify the changes in the gene copy number and in the amounts of heterogeneous nuclear (hn)RNA and mRNA that have occurred during the development of resistance to DFMO in a human myeloma-cell line. This has been performed with the aid of the PCR combined with reverse transcription. The results obtained indicated that the enhanced accumulation of ODC-specific mRNA is directly related to increased amounts of hnRNA and finally to the elevated ODC gene copy number.

EXPERIMENTAL

Cell lines

The parental human Sultan myeloma (IgG) cell line has been described by Ralph (1979). The DFMO-resistant variant cell line was isolated upon exposing the myeloma cells to increasing concentrations of DFMO over a period of several months (Leinonen *et al.*, 1987). At the time of the present analyses, the cells have been grown in the presence of 20 mM-DFMO for several years. The characteristics of this DFMO-resistant Sultan myeloma cell line have been described in detail by Hirvonen *et al.* (1989).

Materials

DFMO was generously given by Centre de Recherche Merrell International (Strasbourg, France). [^{32}P]dCTP (sp. radioactivity > 400 Ci/mmol) was purchased from Amersham International (Amersham, Bucks., U.K.). *Taq* polymerase and avian-myeloblastosis-virus (AMV) reverse transcriptase were obtained from Promega Corp. (Madison, WI, U.S.A.). Oligonucleotide primers were synthesized with a 381A DNA synthesizer (Applied Biosystems, Warrington, Cheshire, U.K.) and the PCRs were carried out in a Hybaid thermal reactor (Hybaid, Teddington, Middx., U.K.). The probe for dot-blot-hybridization analyses was generated by PCR using human ODC cDNA as the template. The final sp. radioactivity of this 636 bp nucleotide fragment was 3.4×10^8 c.p.m./μg.

Design of primers for the PCRs

Two sets of primers were used (Fig. 1). Two oligonucleotides targeted to a sequence of third intron (5′ primer: 5′-GGCTTACATGTCTTGTTATGGAATGTAGAA-3′) and to a sequence of fourth intron (3′ primer: 5′-GCTATCCATATGT-GGCTTAACACGTGG-3′) (Fig. 1) were used to prime the PCR in order to detect genomic DNA and hnRNA for human ODC. For the detection of mature mRNA, another pair of primers were used. The 5′ primer (5′-CCTTCGTGCAGGCAATCTCT-3′) recognized a sequence in exon 7 and the 3′ primer (5′GCT-GCATGAGTTCCCACGCA-3′) recognized a sequence at the junction of exons 10 and 11 of human ODC cDNA (Fig. 1). The design of the 3′ primer prevented any genomic DNA from being amplified.

Abbreviations used: ODC, ornithine decarboxylase; DFMO, α-difluoromethylornithine; hn, heterogeneous nuclear; AMV, avian myeloblastosis virus.

* To whom correspondence should be sent.

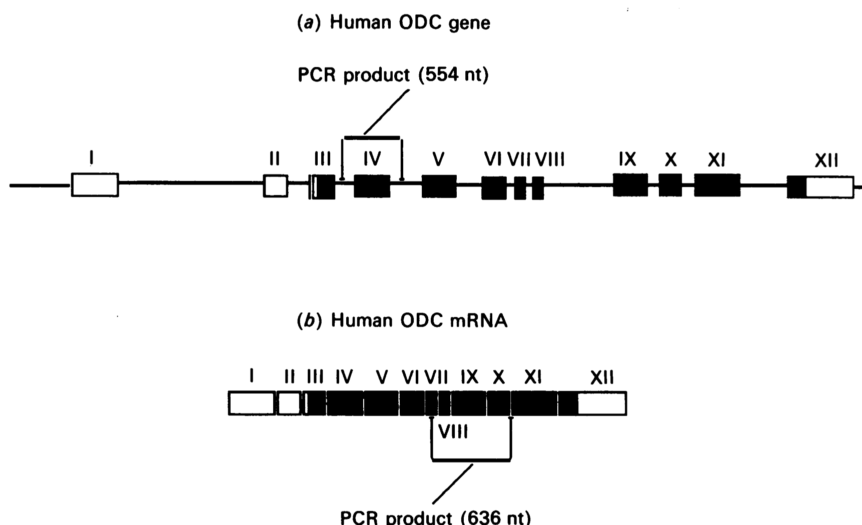

Fig. 1. Design of the PCR primers for the quantification of the gene dosage and the amounts of hnRNA and mRNA of human ODC

(*a*) Exon–intron structure of human ODC gene and the approximate location of the target sequences for PCR primers. (*b*) Location of the exons in human ODC mRNA and the targeting of the PCR primers. The black areas indicate the coding region. The drawings are not strictly to scale.

Isolation of DNA and RNA

The nucleic acids were isolated from 15×10^6 cells at the exponential growth phase. DNA was isolated by the salt precipitation method (Miller *et al.*, 1988) and treated with RNAase. Total RNA was isolated by the guanidinium thiocyanate method (Chomczynski & Sacchi, 1987) and the RNA was dissolved in 50 μl of RNasin in water (1 unit/μl; Promega). Any contaminating DNA was removed by DNAase treatment. The DNAase digestion was carried out in the presence of RNasin (1 unit/μl) and RQ1 RNAase-free DNAase (0.1 unit/μl; Promega) for 30 min at 37 °C. After the digestion RNA was extracted twice with phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.), precipitated with ethanol, dissolved in water containing RNAasin (1 unit/μl) and used for cDNA synthesis. The concentrations of the nucleic acids were measured spectrophotometrically.

cDNA synthesis

The first strand cDNA synthesis was carried out at 42 °C for 60 min in the presence of AMV reverse transcriptase (0.5 unit/μl), RNAasin (1 unit/ml), dNTP (1 mM) and 1 μg of total RNA. mRNA samples were primed with oligo(T) and hnRNA samples with intronic PCR 3′ primer. Immediately after mixing RNA and the reagents, an aliquot was taken from the reaction mixture to be used as a negative control in the PCR.

PCR

The PCR was initiated at 96 °C for 3 min, followed by 28 (mRNA) or 38 (hnRNA) or 32 (DNA) cycles consisting of the following phases: 1 min at 95 °C (denaturation), 1 min at 58 °C (annealing) and 1.5 min at 72 °C (primer extension). Template, genomic DNA or cDNA was pipetted in 2 μl volume. The reaction mixture contained 10 mM-Tris/HCl, pH 9.0, 50 mM-KCl, 2 mM-$MgCl_2$, 0.01 % gelatin, 0.1 % Triton X-100, 0.2 mM-dNTP, 1.25 units of *Taq* polymerase and 15 pmol of the corresponding primers. After the completion of the cycles the samples were left at 72 °C for 5 min. A 15 μl aliquot of the reaction mixture was electrophoresed in 1.5 %-agarose gel, stained with ethidium bromide and bands were revealed under u.v. light. To prepare the probe, plasmid pODC 10/2H (Hickok *et al.*, 1987; 2 ng) was used as a template, and the sample was amplified 35 cycles in the presence of [^{32}P]dCTP (2.5 μM; 400 Ci/mmol).

Scanning of the dot-blot and PCR films

The scanning of the X-ray films (dot-blots) and negative films (PCR products) was performed with a Shimadzu dual-wavelength Chromato Scanner.

RESULTS

As Fig. 2 shows, dot-blot analyses with dilution series indicated that the amount of ODC-specific DNA sequences was at least 100 times higher in the resistant (R) than in the sensitive (S) cells (compare the faint uppermost dot representing 1 μg of DNA in the S column with the 0.01 μg lowermost dot in R column). Similar comparison with the RNA dots (Fig. 2) revealed that the amount of ODC mRNA was at least 100 times higher in the DFMO-resistant (R) cells than in the sensitive (S) cells.

Further dilution experiments carried out with the PCR are depicted in Fig. 3. The use of intron-specific primers in the PCR after the cDNA synthesis allows the detection of hnRNA or pre-mRNA species. A comparison of lanes 1 (1 μg) and 9 (8 ng) in Fig. 3(*a*) indicates that the ODC gene copy number has been increased by a factor of at least 100. In Fig. 3(*b*) the lanes of

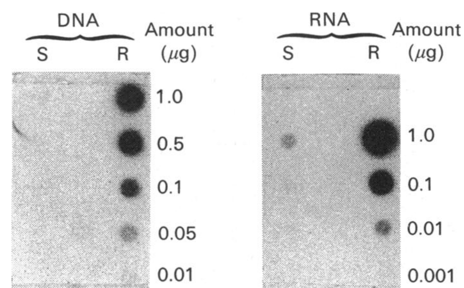

Fig. 2. Dot-blot analysis of the ODC-gene-dosage and amount of mRNA in parental and DFMO-resistant human Sultan myeloma cells

The amounts of nucleic acids used are indicated on the right side of the dots. S, sensitive (parental); R, DFMO-resistant.

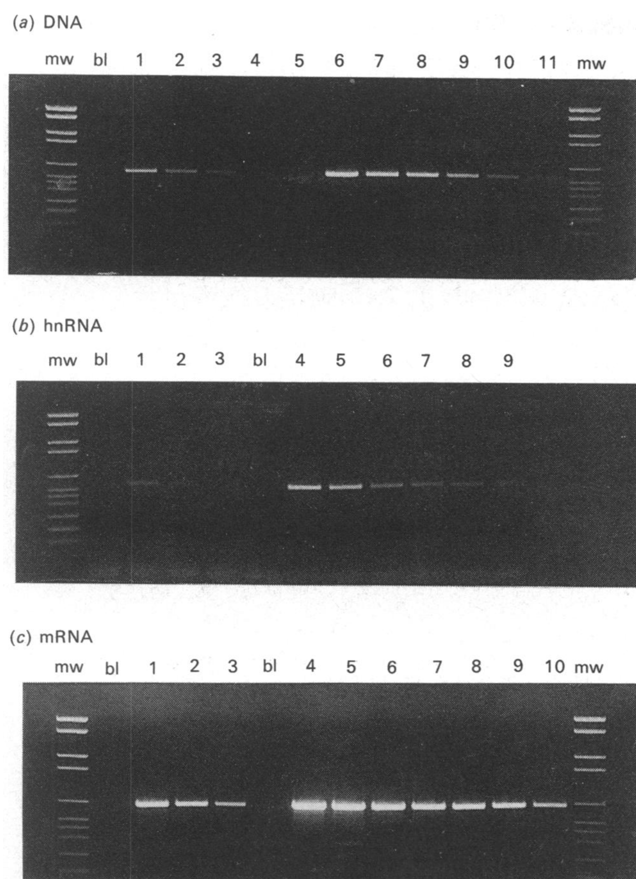

Fig. 3. ODC-gene dosage as revealed by PCR analysis and amounts of ODC-specific hnRNA and mRNA as revealed by the combined reverse transcription/PCR analysis in parental and DFMO-resistant human myeloma cells

(*a*) ODC-gene dosage in parental (lanes 1–5) and DFMO-resistant (lanes 6–11) Sultan myeloma cells. The amounts of DNA template used were as follows: lanes 1 and 6, 1 μg; lanes 2 and 7, 200 ng; lanes 3 and 8, 40 ng; lanes 4 and 9, 8 ng; lanes 5 and 10, 1.6 ng; lane 11, 0.3 ng; mw (here and in *b* and *c*), molecular-size markers (plasmid pBR328 digested with endonucleases *Bgl*I and *Hin*fI); bl, blank (no template). (*b*) Amounts of ODC hnRNA in parental (lanes 1–3) and DFMO-resistant (lanes 4–9) Sultan myeloma cells. The amounts of total RNA used for the first-strand cDNA synthesis were as follows: lanes 1 and 4, 100 ng; lanes 2 and 5, 20 ng; lanes 3 and 6, 4 ng; lane 7, 2 ng; lane 8, 1 ng; lane 9, 0.5 ng; bl, blank (100 ng of total RNA obtained from either parental or DFMO-resistant cells; no cDNA synthesis). (*c*) Amounts of ODC mRNA in parental (lanes 1–3) and DFMO-resistant (lanes 4–10) Sultan myeloma cells. The amounts of total RNA used for the first-strand cDNA synthesis were as follows: lanes 1 and 4, 100 ng; lanes 2 and 5, 20 ng; lanes 3 and 6, 4 ng; lane 7, 2 ng; lane 8, 1 ng; lane 9, 0.5 ng; lane 10, 0.25 ng; bl, blank as in (*b*).

roughly equal intensity are 1 (100 ng of RNA) and 8 (1 ng of RNA), indicating also that the amount of hnRNA species was increased 100-fold in the resistant cells. The same likewise holds true for the amount of the mature mRNA, as revealed by comparing the lanes 1 (100 ng of RNA) and 8 (1 ng).

The results of scanning of the dot-blot X-ray films and PCR photographs (negative films) are presented in Table 1. One may notice that the differences between parental and resistant cells are revealed by the PCR assay were smaller than those obtained with dot-blot analyses. The scatter between individual dot-blot determinations was much larger than that of the PCR. As is also shown in Table 1, the PCR assay indicated that the amounts of ODC genomic sequences, hnRNA and mRNA were increased by

Table 1. Relative amounts of DNA, hnRNA and mRNA in parental and DFMO-resistant human Sultan myeloma cells

The dot-blot X-ray films and the PCR photographs were scanned as described in the Experimental section. The amount of each nucleic acid in the parental cells is set to 1. Results are means ± S.D.; *n* is the number of independent determinations; n.d., not determined.

Nucleic acid	Relative amount as revealed by:	
	Dot-blot	PCR
DNA		
Parental	1 ($n = 4$)	1 ($n = 3$)
Resistant	380 ± 150 ($n = 4$)	130 ± 50 ($n = 3$)
hnRNA		
Parental	n.d.	1 ($n = 7$)
Resistant	n.d.	120 ± 30 ($n = 7$)
mRNA		
Parental	1 ($n = 3$)	1 ($n = 5$)
Resistant	700 ± 300 ($n = 3$)	106 ± 14 ($n = 5$)

the same factor, i.e. about 100-fold. The scanning of the dot-blots (Table 1) showed that the amount of mRNA was about 1.8 times higher than would be expected from the gene dosage.

DISCUSSION

As indicated by the present results, the PCR combined with reverse transcription appears to be an extremely convenient method to study different stages of gene expression. Especially valuable is the fact that, by using this method together with intron-specific primers, it is possible to detect pre-mRNA species and thus get an idea of the transcriptional activity of a given gene. The determination of hnRNA levels existing *in vivo* is an easy alternative to the rather cumbersome nuclear run-on assay. The steady-state levels of hnRNA naturally reflect not only the rate of transcription but also that of processing. Rough estimates indicated that the ratio of ODC hnRNA to mRNA was about 1:100, which is in good agreement with the ratio reported for human thymidine kinase gene (Lipson & Baserga, 1989).

In comparison with dot-blot analyses, the PCR assay gave smaller differences between the parental and overproducer cells. This may be attributable to the number of cycles used, as the sensitivity of the method decreases with increasing number of cycles (Delidow *et al.*, 1989) or to the specificity of the PCR; it detects only sense RNA, and probably not as highly degraded RNAs as the dot-blots do. The same is true also at the DNA level; PCR gives one signal per target, but degradation products may give more signals per target in dot-blot. The dot-blot assays are further complicated by the low basal levels of DNA and mRNA that makes the determination of parental cell levels imprecise. Greater loading of the dots is not possible owing to viscosity or saturation of the membrane capacity. In any event, the method is extremely reproducible, as the reverse transcription/PCR for RNA shows relatively small scatter for independent determinations (Table 1). In comparison with blot analyses, the PCR is also unbeatable as regards the sensitivity and time required to complete the analysis.

It thus appears that the overexpression of ODC in the DFMO-resistant human myeloma cells is solely attributable to the increased gene copy number. This is in contrast with mouse myeloma cells with ODC-gene amplification. In their initial report on mouse ODC-gene amplification, Kahana & Nathans (1984) noticed that ODC-specific mRNA level was much higher than would be expected from the increased gene copy number.

Later on, Katz & Kahana (1989) showed that ODC-gene amplification in mouse myeloma cells was associated with a genetic rearrangement leading to the translocation of ODC gene next to the switch region of the $\gamma 1$ immunoglobulin gene. This rearrangement was presumed to involve also a deletion of a putative silencer sequence at the ODC 5′ flanking region, resulting in a transcriptional activation of the gene (Katz & Kahana, 1989).

Even though human and mouse ODC genes have the same chromosomal localization (human chromosome 2p is equivalent to mouse chromosome 12), our present results, as yet based only on one variant human cell line, do not support the idea that similar genetic rearrangements with a deletion of a transcriptional silencer element has occurred in the case of human ODC-gene amplification.

The skilful technical assistance of Ms. Anne Karppinen and the competent secretarial work of Ms. Taru Koponen are gratefully acknowledged. This work received financial support from the Academy of Finland and from the Finnish Foundation for Cancer Research.

REFERENCES

Alhonen-Hongisto, L., Sinervirta, R., Jänne, O. A. & Jänne, J. (1985) Biochem. J. **232**, 605–607

Alhonen-Hongisto, L., Leinonen, P., Laine, R. & Jänne, J. (1987) Biochem. Biophys. Res. Commun. **144**, 132–137

Chomczynski, P. & Sacchi, N. (1987) Anal. Biochem. **162**, 156–159

Delidow, B. C., Peluso, J. J. & White, B. A. (1989) Gene Anal. Technol. **6**, 120–124

Hickok, N. J., Seppänen, P. J., Gunsalus, G. L. & Jänne, O. A. (1987) DNA **6**, 179–187

Hickok, N. J., Wahlfors, J., Grozat, A., Halmekytö, M., Alhonen, L., Jänne, J. & Jänne, O. A. (1990) Gene **93**, 257–263

Hirvonen, A., Eloranta, T., Hyvönen, T., Alhonen, L. & Jänne, J. (1989) Biochem. J. **258**, 709–713

Kahana, C. & Nathans, D. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 3645–3649

Katz, A. & Kahana, C. (1989) EMBO J. **8**, 1163–1167

Leinonen, P., Alhonen-Hongisto, L., Laine, R., Jänne, O. A. & Jänne, J. (1987) Biochem. J. **242**, 199–203

Lipson, K. E. & Baserga, R. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 9774–9777

McConlogue, L., Dana, S. L. & Coffino, P. (1986) Mol. Cell. Biol. **6**, 2865–2871

Metcalf, B. W., Bey, P., Danzin, C., Jung, M. J., Casara, J. & Vevert, J. P. (1978) J. Am. Chem. Soc. **100**, 2551–2553

Miller, S. A., Dykes, D. D. & Polesky, H. F. (1988) Nucleic Acids Res. **16**, 1215

Ralph, P. (1979) Immunol. Rev. **48**, 107–121

Winqvist, R., Mäkelä, T. P., Seppänen, P., Jänne, O. A., Alhonen-Hongisto, L., Jänne, J., Grzeschik, K.-H. & Alitalo, K. (1986) Cytogenet. Cell Genet. **42**, 133–140

Received 17 December 1990/26 March 1991; accepted 8 April 1991