Volume 227, number 1, 56–60 FEB 05466 January 1988

A protein of *Halobacterium halobium* immunologically related to the v-*myc* gene product

Kamel Ben-Mahrez, Bernard Perbal+, Cecile Kryceve-Martinerie+, Dominique Thierry* and Masamichi Kohiyama

Institut J. Monod, Université Paris VII, 75251 Paris Cedex 05, +Institut Curie, Centre Universitaire, 91405 Orsay and **CEA, IPSN, DPS, SHR, BP 6, 92260 Fontenay aux Roses Cedex, France*

Received 6 November 1987

A 70 kDa protein of *Halobacterium halobium* cross-reacts with an antiserum directed against the v-*myc* gene product of the avian myelocytomatosis virus (MC29). This cross-reaction is in agreement with hybridization studies which indicate that *H. halobium* possesses DNA and RNA sequences homologous to the v-*myc* gene.

v-*myc* gene product; *gag-myc* protein; c-*myc* protein; Southern blot; Western blot analysis; (Archaebacteria)

1. INTRODUCTION

Sequences homologous to the avian myelocytomatosis virus (MC29) v-*myc* oncogene have been found in the genome of many eukaryotes considered to be phylogenetically very distant [1]. Although the exact roles of this oncogene and corresponding proto-oncogene products remain to be established, a growing body of evidence suggests that the expression of c-*myc* might be involved in regulatory processes related to cell division [2] and the DNA synthesis [3].

The c-*myc* polypeptide has been identified by immunological techniques. In avian cells the expression of c-*myc* sequences gives rise to a nuclear 58 kDa polypeptide [4]. In human cells, c-*myc*-specific products, a major 62 kDa and a minor 66 kDa, have been found [5] and shown as being DNA-binding proteins [6].

Our recent finding of positive signals in hybridization between the genome of some archaebacteria and a v-*myb* probe [7], together with the reported homology between the viral *myc* and *myb* oncogenes [8], led us to search for the presence of *myc*-related sequences in *Halobacterium halobium* and for a possible expression of both oncogene-like sequences.

In this report, we describe the purification of a *H. halobium* 70 kDa protein which is immunologically related to the MC29 v-*myc* gene product.

Correspondence address: M. Kohiyama, Institut J. Monod, Université Paris VII, 2, place Jussieu, 75251 Paris Cedex 05, France

2. MATERIALS AND METHODS

2.1. *Strain and culture*

H. halobium CCM2090 was cultured in the complex halophilic medium of Sehgal and Gibbons [9] until an absorbance of 1.4 at 600 nm.

The normal and transformed quail embryo fibroblasts (non-producer clone MC29 Q8) were kindly provided by Dr Catherine Bechade (Institut Curie, Orsay-France) [10].

RP9 is a continuous cell line derived from a RAV-2-induced lymphoma [11] and provided by Dr Simon Saule (Institut Pasteur, Lille-France).

Normal B-lymphocytes were taken from bursa of uninfected one month-old chicken and isolated as described [12].

2.2. *DNA probes*

myc probe [13] and *gag*-specific probe (B2H193) [14] were used.

2.3. *Antibodies*

Polyclonal antibodies raised against a v-*myc* synthetic peptide were purchased from Oncor Inc., USA and anti-rabbit IgG antiserum linked to peroxidase was from Miles.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

2.4. *RNA extraction and blotting*

Total RNA was extracted from exponentially growing cells (*H. halobium*) according to the method of Chirgwin et al. [15]. The blotting is carried out as described in [16].

2.5. *Preparation of DNA-free extracts*

25 g of frozen *H. halobium* cells were resuspended in 25 ml of buffer containing 25 mM Hepes, pH 7.5, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA and 3 M KCl, and broken by Potter homogenizer at 4°C. After centrifugation at 4°C (100000 × *g* for 60 min), the supernatant was withdrawn and adjusted to 11% polyethylene glycol 6000 and 2.3% dextran T-500 (Pharmacia). After gentle stirring for 1 h at 4°C, the mixture was centrifugated at 4°C (5000 × *g* for 10 min) and the upper phase (polyethylene glycol fraction) was removed.

2.6. *Protein determinations*

Protein was determined by the method of Bradford [17], bovine serum albumin was the protein standard.

2.7. *Western blot analysis*

Samples of protein were precipitated by trichloroacetic acid (6%) and the pellet was resuspended, after washing with acetone, in 10 mM sodium phosphate, pH 7.0, 1% SDS and 1% 2-mercaptoethanol and heated at 100°C for 5 min. After electrophoresis in SDS-polyacrylamide gel, the proteins were electrophoretically transferred onto Millipore nitrocellulose at 60 V for 2.5 h at 4°C. The transfer buffer consisted of 25 mM Tris-HCl, pH 8.5, 192 mM glycine and 20% (v/v) methanol. After transfer, the blot was washed for 2 h in buffer X (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.1% Tween 20 and 0.25% gelatin) and incubated overnight at 4°C in buffer X containing the antibodies. Then, the blot was rinsed 3 times for 5 min in buffer X and incubated for 2 h at 4°C with an anti-rabbit IgG antiserum linked to peroxidase in buffer X.

After 3 rinses in buffer X without gelatin, the blot was revealed by incubating for 15 min in buffer containing 15 mg 4-chloro-1-naphthol (Sigma), 5 ml ethanol, 25 ml buffer (50 mM Tris-HCl, pH 7.5, and 200 mM NaCl) and 20 μl H_2O_2 (9%).

2.8. *Preparation of an antiserum directed against the 70 kDa protein*

A rabbit was immunized with 100 μg protein in Freund's complete adjuvant by injection at multiple subcutaneous sites. A booster immunization, containing 100 μg protein in Freund's incomplete adjuvant was administered after 3 weeks. Bleedings were performed 15 days after injection.

3. RESULTS

3.1. *Search for DNA sequences related to* v-myc *in* H. halobium

Purified DNA from *H. halobium* was digested with *Xho*I restriction endonuclease and the resulting fragments were electrophoresed, blotted onto Millipore nitrocellulose and hybridized with nick-translated ^{32}P-labeled probes. As shown in fig.1A, hybridization with the *gag-myc* probe led to the detection of three major fragments (5.0, 3.4 and 2.9 kb). Since the probe that we used contained about 1 kb of the retroviral *gag* gene [13], duplicate blots were hybridized with the *gag*-specific probe (B2H193) derived from MAV 1 proviral DNA [14].

After hybridization with the *gag* probe, a 5 kb fragment was only detected (fig.1A). This demonstrates not only the existence of v-*myc* related DNA sequences but also the presence of *gag*-like DNA sequences in *H. halobium*.

3.2. *Search for RNA sequences related to* v-myc *in* H. halobium

In order to determine whether these sequences are expressed in *H. halobium*, RNA from exponentially growing cells was purified, electrophoresed in formaldehyde-agarose gel and transferred onto Millipore nitrocellulose. The resulting blots were hybridized with the *gag-myc* probe. In addition, a control hybridization was performed with the *gag*-specific probe (B2H193). Two different RNAs (3.6 and 1.8 kb) were detected with the *myc* probe (fig.1B). Since none of these RNAs hybridized with the *gag*-specific probe, they turned out to represent copies of the sequences related to v-*myc*.

3.3. *V*-myc *related protein in* H. halobium

H. halobium extracts deprived of DNA were prepared by disrupting cells with the aid of a Potter homogenizer, eliminating fast sedimenting material, and treating the soluble fraction by the phase partition (dextran T500-polyethylene glycol 6000) technique in the presence of 3 M KCl, enabling the disruption of protein-DNA complexes (see section 2).

Electrophoretic separation of *H. halobium* proteins obtained in this way was carried out in polyacrylamide gel under denaturing conditions. The presence of immuno-complexes was revealed by a peroxidase conjugate anti-rabbit IgG antiserum after transfer onto Millipore nitrocellulose and incubation in the presence of the anti-v-*myc* antiserum. As shown in fig.2A a unique band of 70 kDa was detected with the anti-v-*myc* antiserum.

3.4. *Purification of the 70 kDa protein of* H. halobium

H. halobium DNA free extracts were prepared

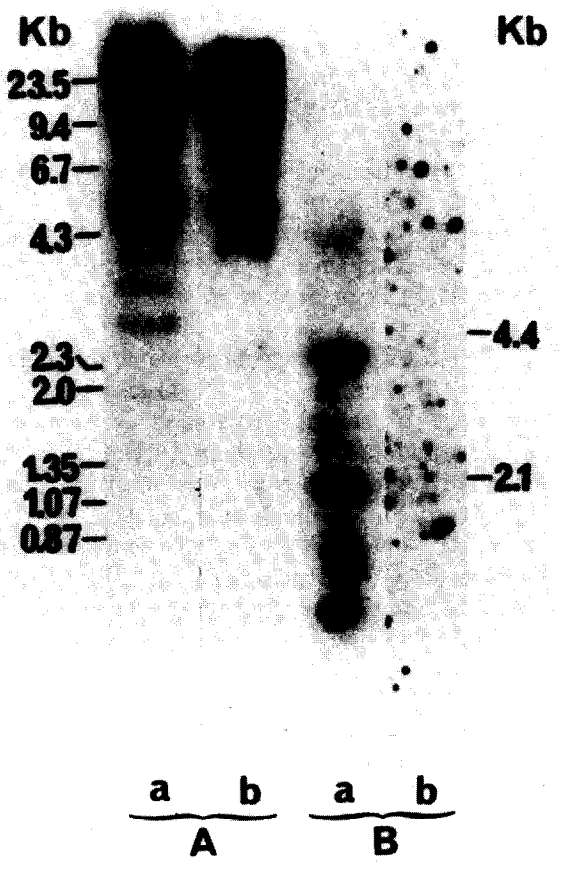

Fig.1. Presence of v-*myc*-like DNA and RNA sequences in *H. halobium*. (A) Samples (10 μg) of purified DNA from exponentially growing *H. halobium* were digested with 50 units of *Xho*I restriction endonuclease and electrophoresed in 1% agarose gel. The resulting fragments were blotted onto Millipore nitrocellulose sheets and hybridized with 15×10^6 cpm of ^{32}P-labeled probes (2×10^8 cpm/μg of DNA) in 10 ml of 35% formamide, 2 × SSC, 5 × Denhardt, 10% dextran sulfate at 42°C for 16 h. Washings were performed at 42°C in 0.1 × SSC [16]. A mixture of *Hin*dIII-digested DNA and *Hae*III-digested ϕX174RF (replicative form) was used as a molecular mass marker. All probes consisted of electroeluted DNA inserts prepared from the corresponding recombinant clones, we have checked that no cross-hybridization was obtained with pBR322-DNA under the used conditions. Lanes: a, *gag-myc* probe; b, *gag* probe. (B) Samples (10 μg) of total RNA from exponentially growing *H. halobium* were fractionated by electrophoresis in formaldehyde-agarose (1%) gel and transferred onto Millipore nitrocellulose sheets [16]. The blots were hybridized as described for A. Chicken ribosomal RNA species (4.4 and 2.1 kb) were used as molecular mass markers. Lanes, a, *gag-myc* probe; b, *gag* probe.

as described in section 2. The polyethylene glycol fraction (2400 mg of protein) was dialysed against buffer containing 20 mM sodium phosphate, pH 7.3, 100 mM KCl and 35% glycerol (v/v), at 4°C until an ionic equivalent of 100 mM KCl was reached. The dialysed fraction was passed through a column of DEAE-cellulose (3 × 15 cm) previously equilibrated with the dialysing buffer except that the glycerol concentration was 10%. The 70 kDa protein was found (by Western blot analysis using the anti-v-*myc* antiserum) in the non-adsorbed and washed fractions (1040 mg protein), which were applied to a column of hydroxyapatite (3 × 10 cm) equilibrated with buffer containing 10 mM sodium phosphate, pH 6.8, 100 mM KCl and 10% glycerol. After washing, the column was eluted with a linear gradient of 0.05–0.4 M sodium phosphate, pH 6.8, in equilibrating buffer (total volume 700 ml) and the 70 kDa protein was eluted around 0.15 M sodium phosphate, pH 6.8. The 70 kDa protein fractions (8 mg protein) were pooled and dialysed, against a buffer containing 20 mM sodium phosphate, pH 6.8, 100 mM KCl and 0.5 mM EDTA, at 4°C until an ionic equivalent of 100 mM KCl was reached, then passed through a column of carboxymethyl cellulose (1 × 6 cm) previously equilibrated with the dialysing buffer. After washing, the column was eluted with a linear gradient of 1–3 M NaCl in dialysing buffer (total volume 40 ml). The 70 kDa protein was eluted around 2 M NaCl (40 μg protein).

At this stage, the 70 kDa protein was electrophoretically pure when analysed in denaturing polyacrylamide gels (fig.2B), and cross-reacted with the anti-v-*myc* antiserum (fig.2C).

3.5. *Polyclonal antiserum against the 70 kDa protein*

100 μg of purified 70 kDa protein were injected into a rabbit in order to obtain an antiserum. As shown in fig.3A, the antiserum obtained after immunization recognizes specifically the same 70 kDa peptide as recognized by the anti-v-*myc* antiserum.

In quail cells transformed by MC29 virus, a viral fusion protein 110 kDa *gag-myc* is synthesized [18]. In order to confirm that the 70 kDa protein is related to the viral *myc* protein, we have tested the anti-70 kDa antiserum by Western blot analysis using extracts of quail cells transformed by MC29 virus. Fig.3B shows that the anti-70 kDa

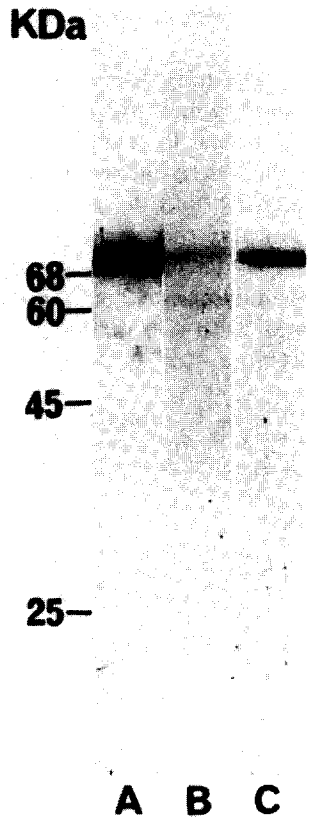

Fig.2. Western blot detection of the 70 kDa protein from *H. halobium* and its purification. (A) A sample of the polyethylene glycol fraction corresponding to 100 μg protein was electrophoresed in SDS-10% polyacrylamide gel. After transfer onto Millipore nitrocellulose, the proteins were incubated with the anti-v-*myc* antiserum (1/20 dilution of stock solution) overnight at 4°C. After washing, the sheets were incubated for 2 h at 4°C with an anti-rabbit IgG antiserum linked to peroxidase. The position of various molecular mass markers (Boehringer) are shown on the left-hand side. (B) 2 μg protein from carboxymethyl cellulose column fractions containing the 70 kDa protein were electrophoresed in SDS-10% polyacrylamide gel and Coomassie blue stained. (C) A Western blot of the gel in B using the anti-v-*myc* antiserum as described in A.

antiserum recognizes a 110 kDa peptide corresponding to the *gag-myc* fused protein in extracts of quail cells transformed by MC29 but not in normal cells. Then we examined whether the avian c-*myc* protein is also recognized by our antiserum using the extract of RP9 cells which produce large amounts of the c-*myc* protein [11]. Western blot analysis revealed a peptide of 58 kDa corresponding to the avian c-*myc* gene product, this peptide was not detected in normal B-lymphocytes (fig.4).

4. DISCUSSION

Archaebacteria are prokaryotes which share several characteristics specific to eukaryotes [19]. Here we have reported the finding of another eukaryotic characteristic: the presence in the genome of an halophilic archaebacteria (*H. halobium*) of DNA fragments which hybridize with a v-*myc* probe. We have isolated a clone of *H. halobium* DNA which hybridizes with the v-*myc* probe, and partly determined the nucleotide sequence (600 bp). We found a significant homology between the v-*myc* protein and the *H. halobium* clone deduced amino acid sequence [20]; firstly, it

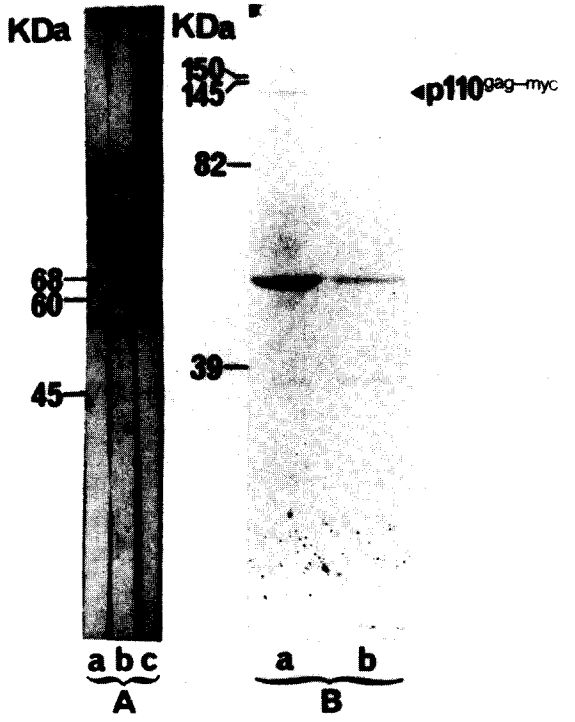

Fig.3. Western blot test of the anti-70 kDa antiserum. (A) 100 μg protein from polyethylene glycol fractions were separated in SDS-10% polyacrylamide gel, transferred onto Millipore nitrocellulose and incubated as described for fig.2A with: (a) control serum (1/100 dilution); (b) anti-v-*myc* antiserum (1/20 dilution); (c) anti-70 kDa antiserum (1/100 dilution). (B) Approx. 10^6 quail cells were lysed in 10 mM sodium phosphate, pH 7, 1% 2-mercaptoethanol, 1% SDS and 1 mM phenylmethylsulfonyl fluoride and heated at 100°C for 5 min. Samples of 50 μg protein from quail cells infected by MC29 virus (a) or control quail cells (b) were subjected to electrophoresis in SDS-7% polyacrylamide gel. The Western blot analysis using the anti-70 kDa antiserum (1/100 dilution) was as described for fig.2A.

showed 25.5% homology with the v-*myc* protein, and secondly it contained a similar amino acid sequence to one of the conserved regions shared by the oncogenes *myc, myb* and adenovirus E1a [8]. Besides DNA hybridization, we have also found a 70 kDa protein in *H. halobium* which is recognized by an anti-v-*myc* antiserum. A close immunological similarity has clearly been demonstrated by the fact that the polyclonal antiserum against the 70 kDa protein reacted with the viral *gag-myc* and avian c-*myc* proteins. Although no evidence is yet available concerning direct correspondence between our DNA clone and the *myc*-like protein, these two elements support the idea of archaebacteria being closer to eukaryotes than prokaryotes.

In this study, we have found a DNA fragment from *H. halobium* which too hybridized with the retroviral *gag* gene. In exponentially growing bacteria, only the v-*myc*-like sequence is expressed: we have detected by hybridization two RNAs (3.6 and 1.8 kb). No *myb* (not shown)- or *gag*-specific RNA could be detected. Accordingly, using Western blot analysis, we could not find cross-reacting material with two different anti-*myb* antisera (not shown).

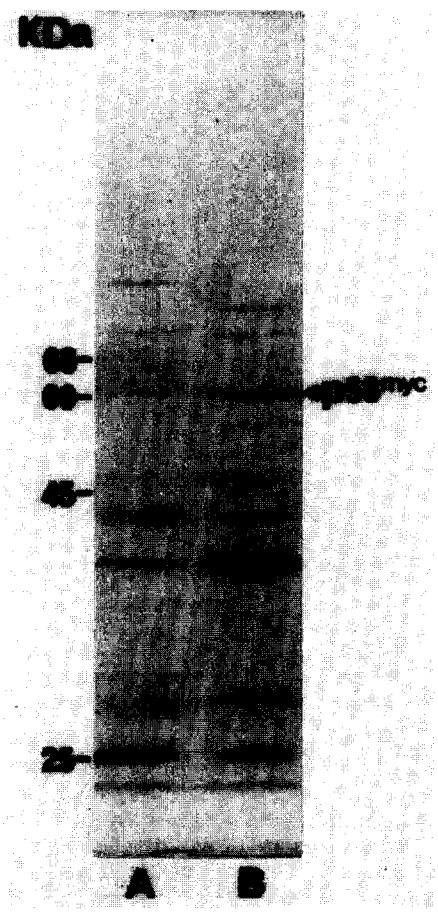

Fig.4. Cross-reaction between the anti-70 kDa antiserum and the avian c-*myc* protein. 50 μg protein from B-lymphocyte (A) and RP9 cell (B) extracts were subjected to electophoresis in SDS-10% polyacrylamide gel. The Western blot analysis using the anti-70 kDa antiserum (1/200 dilution) was as described in the legend of fig.2A. The cell extracts were prepared as described for fig.3B.

Acknowledgements: This work was supported by grants from CNRS (ATP960067, ATP902041) and ARC (Association pour la recherche sur le cancer) to B.P. and M.K.

REFERENCES

[1] Roussel, M., Saule, S., Lagrou, C., Rommens, C., Beug, H., Graf, T. and Stehelin, D. (1979) Nature 281, 452–455.
[2] Campisi, J., Gray, H.E., Pardee, A.B., Dean, M. and Sonenshein, G.E. (1984) Cell 36, 241–247.
[3] Studzinski, G.P., Brelvi, Z.S., Feldman, S.C. and Rosemary, A.W. (1986) Science 234, 467–470.
[4] Alitalo, K., Ramsay, G., Bishop, J.M., Pfeifer, S.O., Colby, W.W. and Levinson, A.D. (1983) Nature 306, 274–277.
[5] Ramsay, G., Evan, G.I. and Bishop, J.M. (1984) Proc. Natl. Acad. Sci. USA 81, 7742–7746.
[6] Persson, H. and Leder, P. (1984) Science 225, 718–721.
[7] Perbal, B. and Kohiyama, M. (1985) CR Acad. Sci. 300, 177–180.
[8] Ralston, R. and Bishop, J.M. (1983) Nature 306, 803–806.
[9] Sehgal, S.N. and Gibbons, N.E. (1960) Can. J. Microbiol. 6, 165–169.
[10] Bister, K., Hayman, M.J. and Vogt, P.K. (1977) Virology 82, 431–438.
[11] Hayward, W.S., Neel, B.G. and Astrin, S.M. (1981) Nature 290, 475–480.
[12] Böyum, A. (1968) J. Clin. Lab. Invest. 21, suppl.97, 77–89.
[13] Hamlyn, P.H. and Rabbitts, T.H. (1983) Nature 304, 135–139.
[14] Soret, J., Kryceve-Martinerie, C., Crochet, J. and Perbal, B. (1985) J. Virol. 55, 193–205.
[15] Chirgwin, J.M., Przybyla, A.E., McDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[16] Perbal, B. (1984) A Practical Guide to Molecular Cloning, Wiley, New York.
[17] Bradford, M.M. (1976) Anal. Biochem. 72, 248–255.
[18] Ramsay, G., Hayman, M.J. and Bister, K. (1982) EMBO J. 1, 1111–1116.
[19] Zilling, W., Schnabel, R. and Stetter, K.O. (1985) Curr. Top. Microbiol. Immunol. 114, 1–18.
[20] Ben-Mahrez, K., Sougakoff, W., Nakayama, M. and Kohiyama, M. Arch. Microbiol., in press.