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FUNCTIONAL ANALYSIS OF THE HUMAN CALCYCLIN GENE PROMOTER IN A PANEL OF HUMAN MELANOMA CELL LINES

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By comparing two subsequent human tumor stages we previously described calcyclin as a new potential melanoma associated neoplastic progression marker positively linked with metastasis. In this study the calcyclin expression levels in a representative panel of human melanoma cell lines were correlated with the occurrence of DNase I hypersensitive (DH) regions and potential enhancer elements in a 6 kb genomic fragment spanning the human calcyclin gene. Examination of the chromatin structure of the transcription unit revealed no qualitative differences in DH sites within the panel of tested human melanoma cells, but especially the sequences around the transcription start site and a 1.5 kb upstream region appeared more accessible to the nuclease in frequently (BLM, MV3) as compared to poorly (530, 1F6) metastasizing cells. The genomic fragments that harbor one or more DH sites were subjected to functional analysis by luciferase reporter gene assays. Thus, an enhancer element was detected between 361 and 167 bp upstream of the transcription start site. This enhancer displayed equal activating potential (2-3 fold) both in weakly and in frequently metastasizing cells and was apparently recognized by transcription factors present in both types of human melanoma cell lines. We conclude that, in addition to a slight amplification of the encoding gene, the elevated calcyclin mRNA levels are only reflected in a selectively increased accessibility of the chromatin structure to DNaseI in metastasizing melanoma cells.

Calcyclin is a small calcium-binding protein (10.5 kD) belonging to the S100 family containing two calcium-binding structures, the so called EF hands. It was identified as a differentially expressed mRNA in serum-stimulated and quiescent fibroblasts [1,2] and was originally isolated from G_1-specific temperature sensitive mutants of baby Syrian hamster kidney cells [3].

Although the function of calcyclin is still unknown, several observations can be made: the calcyclin gene was induced by serum, platelet-derived growth factor, or epidermal growth factor [1,4,5]. The gene product was shown to bind both zinc and calcium [6] which indicated that it is involved in processes related to signal transduction.

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Secondly, aberrant expression of calcyclin was reported for human myeloid leukemias [1], and overexpression was found to occur in thick melanomas as compared to thin ones [7] and in *ras*-transformed metastatic NIH/3T3 cells [8]. Finally, the calcyclin protein was reported to associate with other proteins. Murphy *et al.* [9] isolated calcyclin as a prolactin receptor associated molecule. Interactions in a calcium-dependent manner were reported for several members of the annexin family, among which annexin II, VI, and XI [10,11,12]. Associations with glyceraldehyde-3-phosphate dehydrogenase [12], and sialic acid [13] were also described.

By applying the differential hybridization technique to two subsequent stages of melanocytic tumor progression to identify new progression markers for human cutaneous melanoma, several differentially expressed clones were isolated, one of which appeared to be coding for calcyclin [14]. The expression of calcyclin was at least ten times elevated in the highly metastatic human cell lines BLM and MV3 as compared to the poorly metastatic cell lines 530 and 1F6 (see Figure 1A). Correspondingly, a slight amplification of the calcyclin gene in the highly metastatic cell lines was reported although this could not directly account for the differences at the RNA level [14]. The present study was undertaken to gain more insight in the regulation of the human calcyclin gene. With this purpose, we examined the expression profile in a number of human tissues and analyzed flanking sequences of the calcyclin promoter for enhancer activity in a panel of human melanoma cell lines with different metastatic behavior.

MATERIALS AND METHODS

Melanoma cell lines. Human melanoma cell lines MV3, BLM, 530, and 1F6 [15,16] were grown as monolayers on Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, New York) supplemented with 10% fetal calf serum (Gibco Laboratories), glutamine (2 mM), penicillin G (100 units/ml), streptomycin (100 μg/ml), and pyruvate (1 mM). Within this panel of cell lines, 1F6 and 530 represent the poorly metastasizing cell lines, with a metastasis frequency of less than 10% three months after subcutaneous inoculation into nude mice. The cell lines BLM and MV3 represent the highly metastatic phenotype, with over 50% metastasis frequency.

Isolation and analysis of RNA. Total cellular RNA was isolated using the lithium-urea procedure as described by Auffray and Rougeon [17]. Ten micrograms of total RNA were glyoxylated [18], size fractionated on 1% agarose gels, and transferred to Hybond N-plus filters (Amersham, England). Hybridization was performed according to Church and Gilbert [19] with the addition of 0.1 mg denatured herring sperm DNA/ml of hybridization mixture. To confirm that equal amounts of RNA were loaded in each lane, the blots were hybridized afterwards to a 28S ribosomal probe.

Mapping of DNase I hypersensitive sites. Nuclear isolations, DNase 1 digestions and chromosomal DNA purifications were all performed as described by Dirks *et al.* [20]. Purified DNA (100 μg samples) was digested to completion with *Eco*R1 (Gibco Laboratories), purified by phenol and chloroform extractions, precipitated, and dissolved

in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). DNA samples (50 μg) were electrophoresed in vertical 1% agarose gels (6 mm thick) and transferred to Hybond N-plus filters. The *Hin*dIII fragments of phage lambda (400 ng/lane) and the *Hae*III fragments of phage ϕX174 (200 ng/lane) were added to each sample as internal molecular size marker. DNA was hybridized overnight in 0.75 M NaCl, 75 mM sodium citrate, 0.1% SDS, 0.1% Ficoll, 0.1% BSA, 0.1% polyvinylpyrrolidine, 100 μg/ml denatured herring sperm DNA at 68°C with ^{32}P-labeled DNA probes. Filters were washed at 68°C in 15 mM NaCl, 1.5 mM sodium citrate, 0.1% SDS and exposed to Kodak X-Omat AR-5 films.

DNA probes. The human calcyclin gene, a 6 kb *Eco*RI fragment cloned in pUC9, was a generous gift from Dr. R. Baserga (Temple University Medical School, Philadelphia). After subcloning the 5' 0.5 kb *Eco*RI-*Bam*HI and 3' 0.4 kb *Bam*HI-*Eco*RI fragments (see figure 4) from the human calcyclin gene, they were used as probes in mapping the DNase I hypersensitive regions. pMW1 is a pUC18 derived vector containing the entire calcyclin coding region in a 362 bp cDNA fragment [14].

Reporter gene constructs. For progressive deletions of the 5'-flanking region, the 3.0 kb *Bam*HI fragment of human calcyclin gene containing the first exon, was digested with *Sma*I, *Xho*I, *Sph*I, *Nco*I and *Sca*I. The recovered *Sma*I-*Bam*HI (-167/+134) *Xho*I-*Bam*HI (-361/+134), *Sph*I-*Bam*HI (-588/+134), *Nco*I-*Bam*HI (-1194/+134), *Sca*I-*Bam*HI (-1370/+134) fragments and the 3.0 kb *Bam*HI fragment containing the first exon, were filled in and ligated into the *Bgl*II site of the promoterless luciferase gene vector pGL_2basic (Promega, Madison, WI).

Electropermeations and luciferase assays. Supercoiled plasmid DNA was purified by CsCl gradient centrifugation [21] and quantitated by means of both EtBr staining and spectrophotometric measurements. Cells were washed three times with PBS. 2 x 10^7 cells were mixed with 10 μg pCH110 (Pharmacia Sweden) and 10 pmol luciferase construct in 500 μl PBS. After an incubation of 10 min on ice, cells were electropermeated in a 1 ml electroporation cuvette (Eurogentec, Belgium) by a 2000 volt (530 and 1F6) or 1500 volt (MV3 and BLM) pulse from an ISCO type 494 power supply, so that cell survival was 60-70 %. Cells were incubated on ice for 10 min and replated on 10 cm petridishes (Nunc). 48 h after electroporation, cells were lysed in 150 μl of cell lysis buffer (25 mM Bycine pH 7,8, 0,05% Tween-20, 0,05% Tween-80) for 10-15 min at room temperature and cleared by centrifugation. The protein content of each lysate was determined in a protein assay (Biorad, Richmond, CA) and fixed amounts of protein were tested for ß-galactosidase activity [22]. For each cell line in individual experiments, amounts of protein corresponding to equal ß-galactosidase activity were used for luciferase activity assays. 5-15 μg of cell extract, adjusted to 20 μl total volume with cell culture lysis buffer were mixed with 100 μl assay reagent (20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2.5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM dithio-threitol, 270 μM coenzyme A, 470 μM luciferin, 530 μM ATP at room temperature) after which the activity was measured for 1 min in a Beckman model 2800 liquid scintillation counter. The luciferase activities were expressed as square root of measured counts per minute (cpm) minus background cpm.

RESULTS

Expression of calcyclin in human organs. As shown in Fig. 1A the 0.6 kb long calcyclin mRNA is expressed at highly variable levels in the human melanoma cell lines used in this study. Nuclear run-on assays confirmed that the transcription activity was higher in MV3 than in 1F6 (unpublished results). To obtain further clues about the regulation of calcyclin expression, we determined its expression profile in normal human

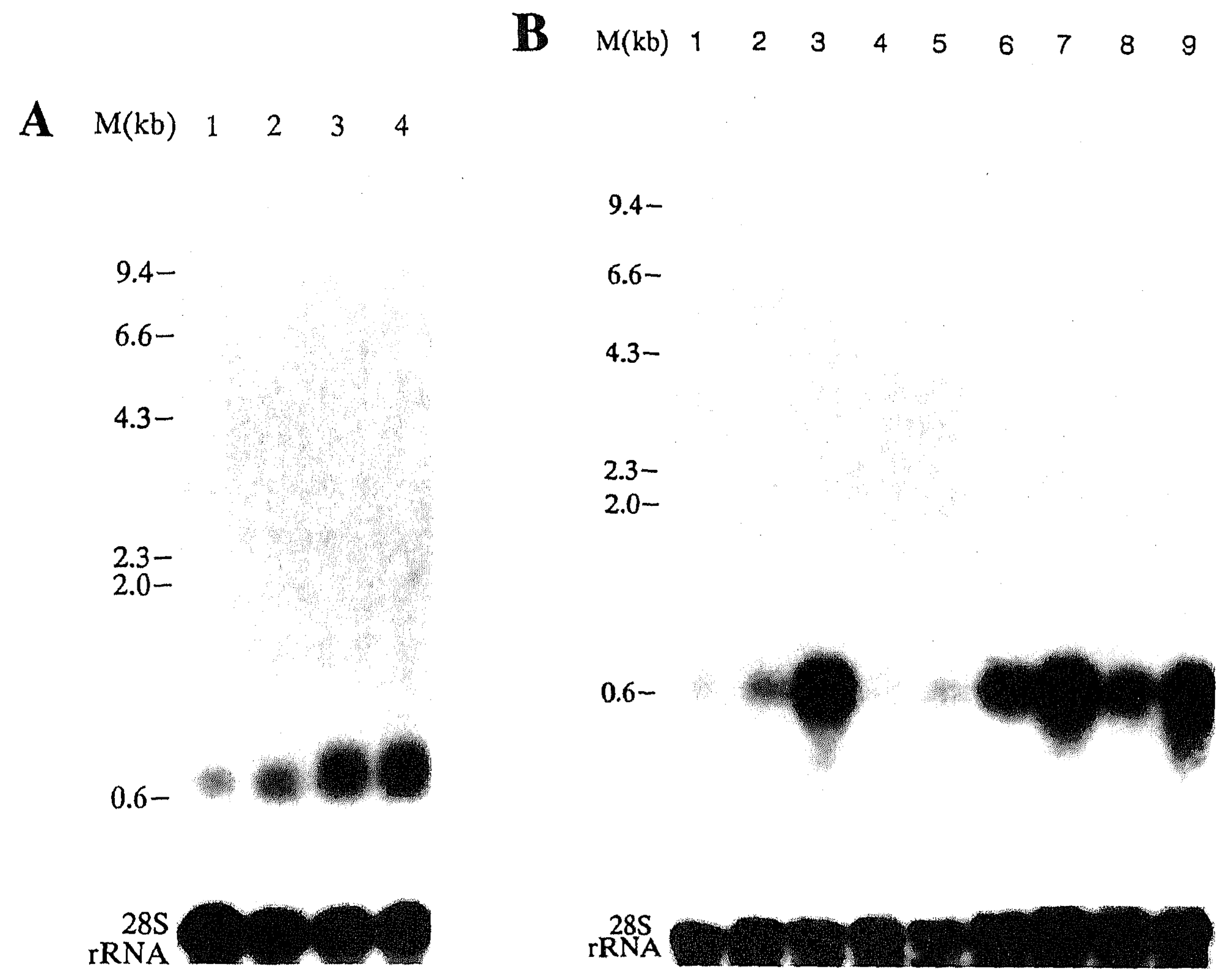


Figure 1. *Northern blot analysis calcyclin mRNA in a panel of human melanoma cell lines (A) and in normal human organs (B).* Ten μg of total RNA was loaded in each lane. pMW1 was used as a molecular probe. The molecular weight marker was lambda DNA digested with restriction enzyme *Hin*dIII; a 28S ribosomal probe was used for the control hybridization.
Panel A: Lane 1: 530; lane 2: 1F6; lane 3: BLM; lane 4: MV3.
Panel B: Lane 1, kidney cortex; lane 2, kidney medulla; lane 3, lung; lane 4, muscle; lane 5, liver; lane 6, placenta; lane 7, spleen; lane 8, prostate; lane 9, intestines.

organs using northern blot analysis (Fig. 1B). highest expression was found in spleen, lung and intestines, followed by prostate and placenta. Weak signals could be seen in liver, muscle, kidney cortex and medulla.

Location and function of regulatory elements for the human calcyclin gene. To map regulating elements we examined the calcyclin gene in the highly metastatic (MV3, BLM) and the poorly metastatic (1F6, 530) cell lines for the presence of DNase I hypersensitive (DH) sites. Using two probes, one hybridizing at the upstream extremity of a 6.0 kb EcoRI genomic fragment (Fig. 2A) and the other at the downstream extremity (Fig. 2B), ten DH sites were detected around the three exons long calcyclin gene in the four human melanoma cell lines. The most prominent DH region included the calcyclin gene promoter and extended from -0.3 to +0.3 kb relative to the transcription start site.

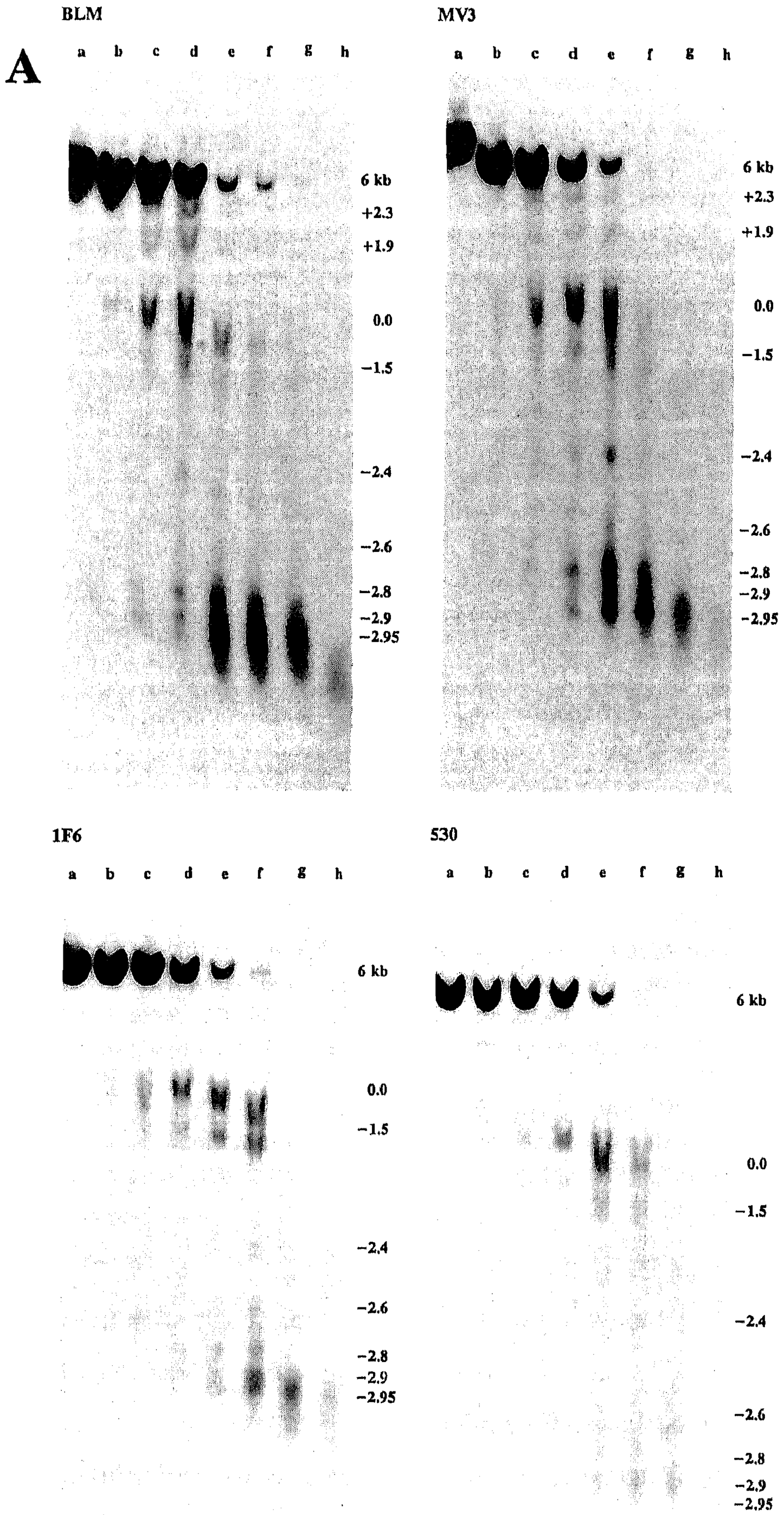


Figure 2. Mapping of the DNase I hypersensitive sites in the 6.0 kb EcoRI calcyclin gene fragment. Nuclei from the indicated cells were treated with increasing amounts of DNase I. Chromosomal DNA (50 μg/lane) was digested with *Eco*RI and subjected to Southern blot analysis. The probes used were the 5' 0.5 kb *Eco*RI-*Bam*HI (panel A) or the 3' 0.4 kb *Bam*HI-*Eco*RI (panel B) fragment. DNase I concentrations were: a, 0 u/ml; b, 64 u/ml; c, 128 u/ml; d, 256 u/ml; e, 512 u/ml; f, 1024 u/ml; g, 2048 u/ml; h, 4096 u/ml. By using endogenous markers for DNA fragment length we established the positions of the DH sites relative to the transcription start site.

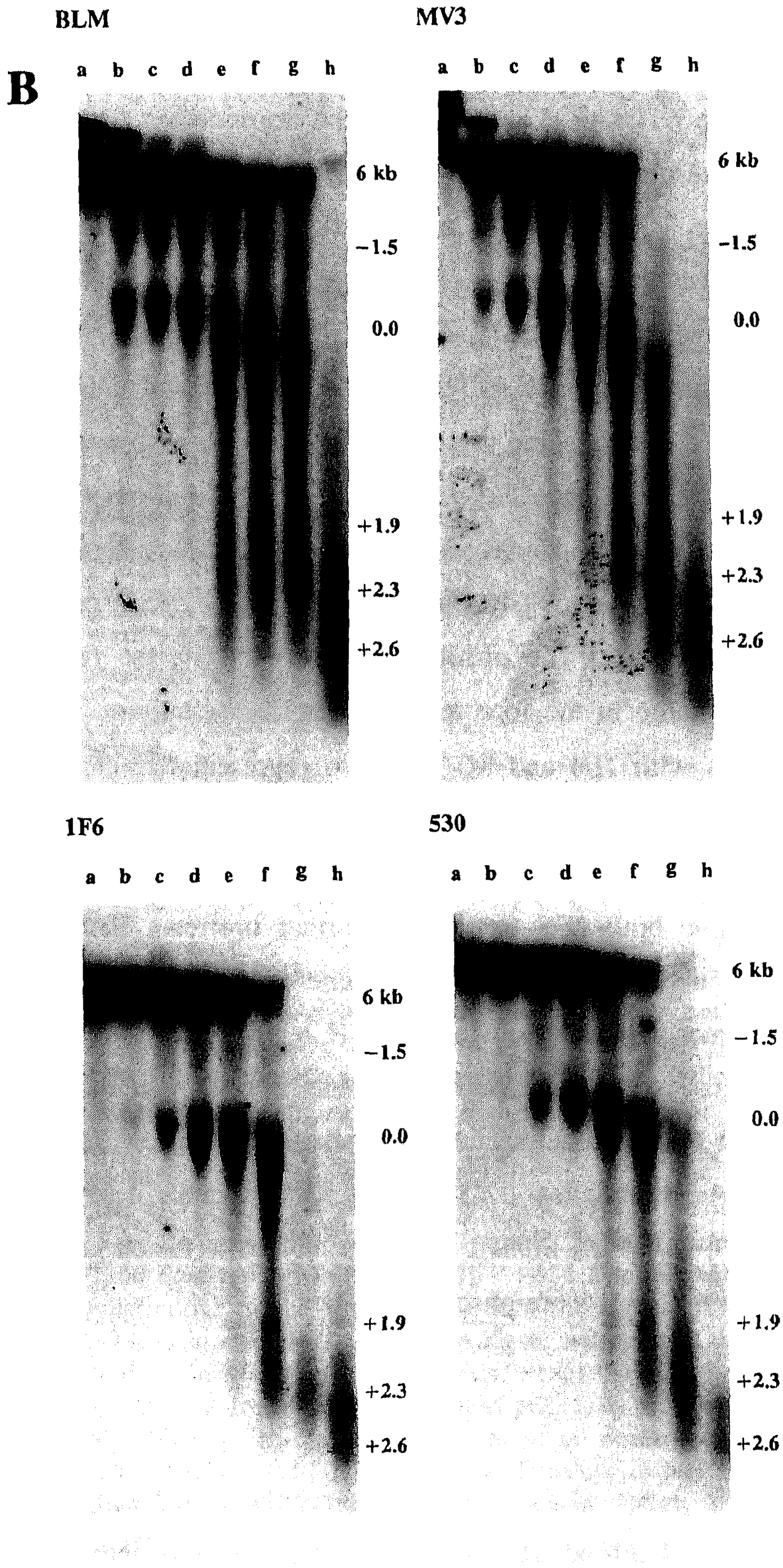


Figure 2 – *Continued*

In MV3 and BLM this region became apparent at lower DNaseI concentrations than in 1F6 and 530. A second DH region was located at -1.5 kb upstream of the transcription start and appeared at the same DNaseI concentrations as the promoter region in MV3 and

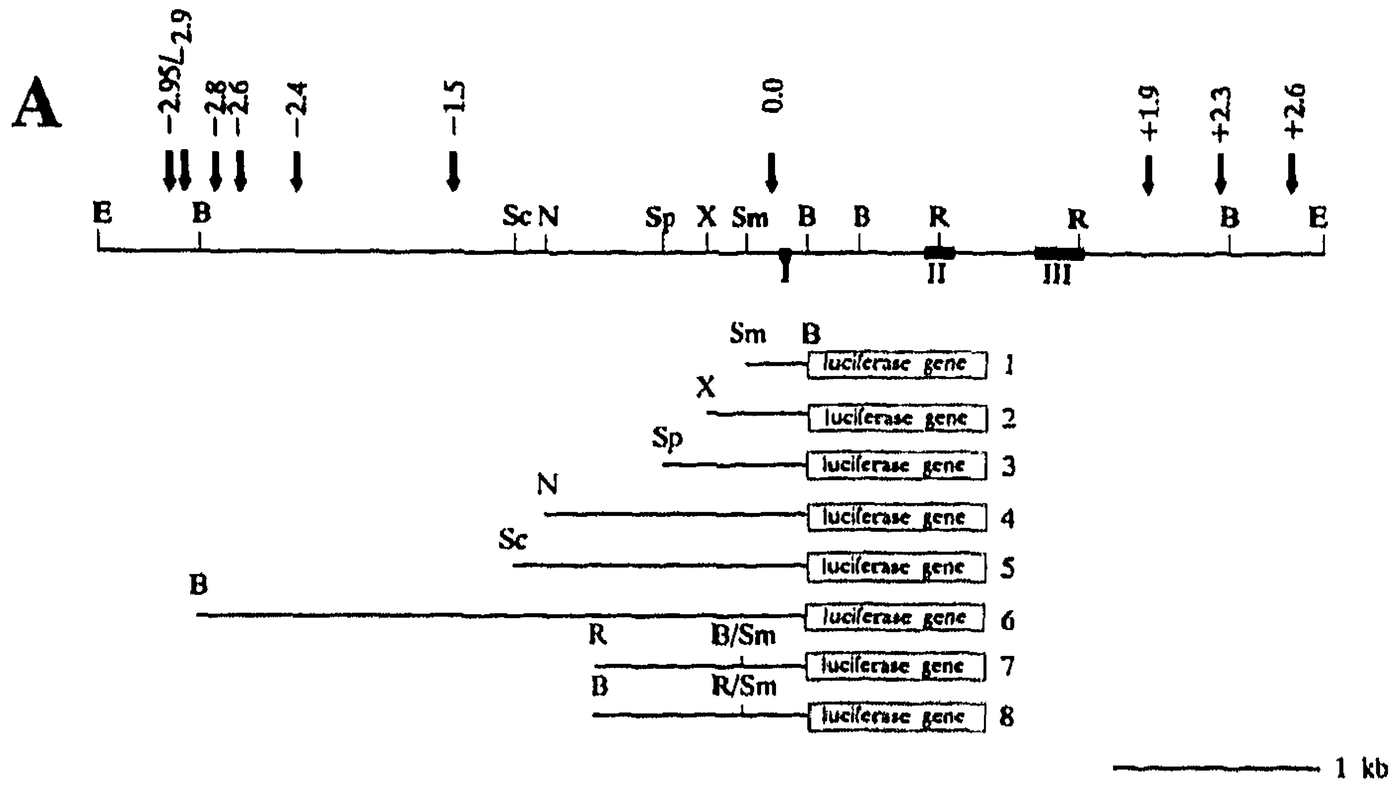
BLM, while it was virtually absent in 1F6 and 530 (Fig. 2B). The other DH sites appear only at higher DNaseI concentrations and may be less important for transcriptional control (see also below). The DH sites downstream of the transcription start (+1.9, +2.3, +2.6 kb) may also be the result of transcription termination.

To study the possibility that the marked differences in calcyclin mRNA expression are modulated by variations in the levels of transactivating factors in human melanoma cell lines, we performed a functional analysis of the calcyclin promoter element including the relevant flanking regions as defined by the presence of DH sites. Progressively deleted sequences of the 5'-flanking region of the calcyclin gene were fused to the coding sequence of the luciferase gene (Fig. 3A). The truncated promoter element constructs were transiently transfected by electroporation into the four melanoma cell lines. In each cell line the activity of the -167/+134 bp SmaI-BamHI calcyclin promoter fragment was arbitrarily defined as 100%. The profiles of relative luciferase reporter gene activities were essentially the same in the four tested melanoma cell lines. The experiments were repeated three times with 1F6 and MV3 as two representative cell lines (Fig. 3B). We observed a 2-3 fold increase in luciferase reporter activity when the -167/+134 SmaI-BamHI fragment was extended upstream to the -361/+134 XhoI-BamHI fragment. This effect was detected in both 1F6 and MV3. Further upstream extensions hardly induced additional changes in the luciferase reporter gene expression in the cell lines. This was also true for construct 5 which included the DH region at -1.5 kb (Fig. 3A). Constructs in which the orientation of the various fragments upstream of the -167/+134 fragment were reversed (anti-sense) were inactive. We also tested the +1366/+2086 *Rsa*I-*Bam*HI fragment which harbors DH sites +2.5 and +1.85 by cloning it upstream (sense and anti-sense) of the -167/+134 *Sma*I-*Bam*HI calcyclin/luciferase fusion gene. As can be seen in fig. 3B no significant effects were observed.

DISCUSSION

The calcyclin gene, which is cell cycle-regulated and can be induced by several growth factors, was identified as a differentially expressed gene when comparing two subsequent stages of melanocytic tumor progression. Calcyclin mRNA expression positively correlated with metastatic potential of the human melanoma cell lines [14].

To obtain clues about its regulation, we examined the human tissue distribution of calcyclin at the mRNA level. High calcyclin expression was found in lung, spleen and intestines, and a moderate expression in prostate and placenta. In contrast, calcyclin mRNA expression was low in rat spleen [14,23]. Although we postulated before that a



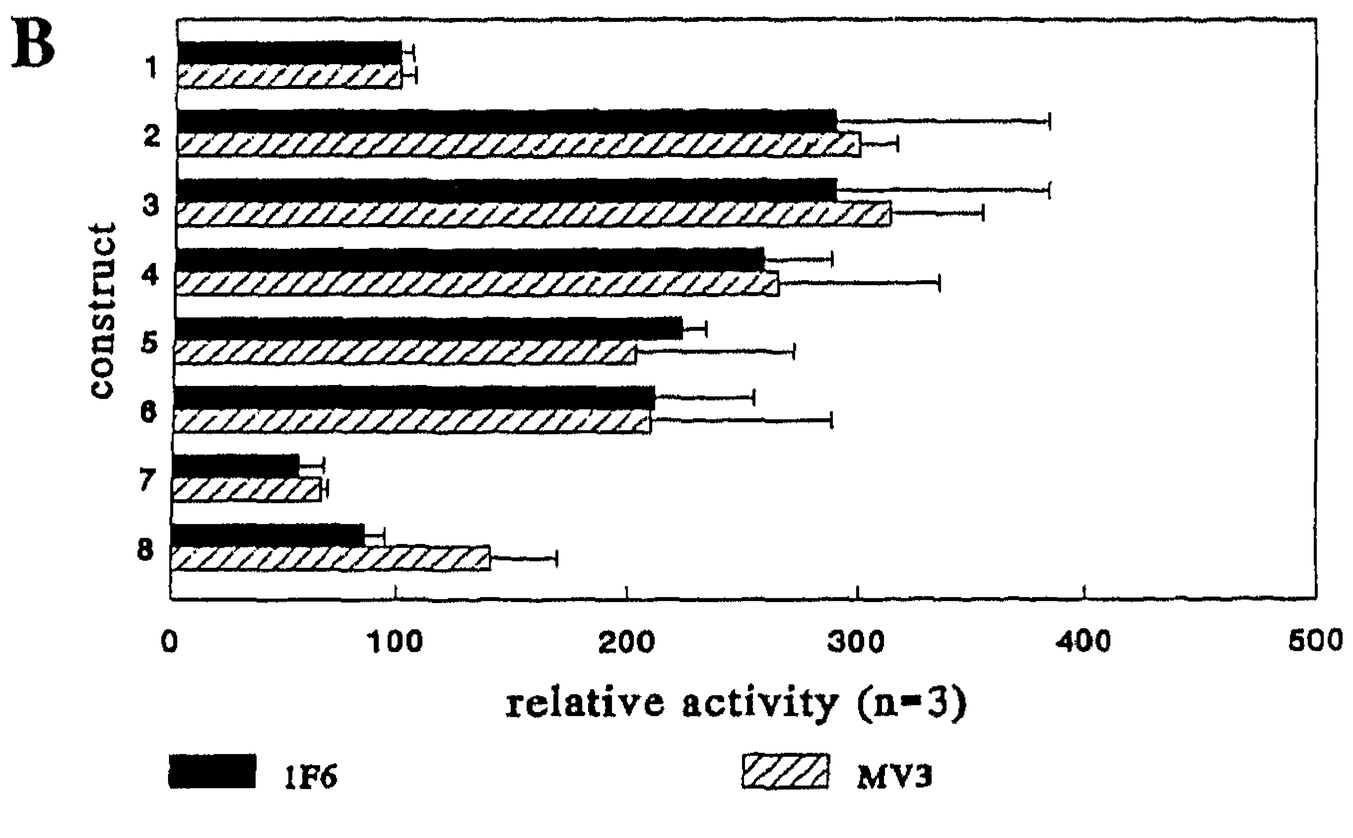


Figure 3. Schematic overview (A) and relative promoter/enhancer activity (B) of the calcyclin/luciferase gene fusion constructs.
Panel A: DH sites in the 6.0 kb *Eco*RI calcyclin gene fragment are indicated by arrows. The three exons are given as boxes. E, *Eco*RI; B, *Bam*HI; Sc, *Sca*I; R, *Rsa*I; Sp, *Sph*I; X, *Xho*I; Sm, *Sma*I; N, *Nco*I; R, *Rsa*I. Calcyclin constructs placed upstream of the luciferase reporter gene were: 1, -167/+134 *Sma*I-*Bam*HI; 2, -361/+134 *Xho*I-*Bam*HI; 3, -588/+134 *Sph*I-*Bam*HI; 4, -1194/+134 *Nco*I-*Bam*HI; 5, -1370/+134 *Sca*I-*Bam*HI; 6, -3.0 kb/+134 *Bam*HI-*Bam*HI; 7, +1366/+2086 *Rsa*I-*Bam*HI in front of -167/+134 *Sma*I-*Bam*HI; 8, +2086/+1366 *Bam*HI-*Rsa*I in front of -167/+134 *Sma*I-*Bam*HI.
Panel B: Transient transfections of the fusion constructs into the human melanoma *cell lines MV3 and 1F6*. Electropermeations, ß-galactosidase assays, and luciferase assays were performed as described in Materials and Methods. Results were normalized to mean ß-galactosidase activity obtained from co-electroporated pCH110. For background corrections we used the promoterless pGL_2-control. Activities of all constructs are indicated as values relative to the activity of the -167/134 *Sma*I-*Bam*HI calcyclin/luciferase gene fusion construct. All data are mean values from three independent electroporations. Standard deviations are indicated by error bars.

high calcyclin mRNA expression might be ascribed to expression in smooth muscle cells present in these organs, a high expression in organs such as the intestines, placenta and the lungs can well be explained by the presence of the lining epithelium which is involved in the secretion of mucus or other substances. Data on protein expression of calcyclin showed that in some human organs such as muscle and placenta, calcyclin protein was

present in fibroblasts, whereas in the majority of other human organs calcyclin was also found in different kinds of epithelial cells [24]. However, calcyclin protein was also detected in other cell types, notably melanocytes [14,25]. Recently, calcyclin was reported to induce insulin secretion in permeabilized pancreas cells in a calcium-dependent manner [26]. Calcyclin expression was also reported at sites of exocytosis [27]. Its connection with the annexins which promote the fusion of membranes is therefore intriguing [10,11,12,28]. The mRNA expression pattern in human organs is in line with a role for calcyclin in secretion pathways.

Since the increased mRNA expression of calcyclin in the highly metastasizing melanoma cell lines could only partially be explained by gene amplification (at the most twofold between cell lines 1F6 and MV3), we sought additional explanations for the elevated mRNA levels. Examination of the chromatin structure of the human calcyclin transcription unit present on a 6.0 kb EcoRI fragment indeed revealed two regions (DH sites 0.0 and -1.5 kb) with an enhanced sensitivity to the nucleolytic action of DNaseI in the highly metastatic cells. By contrast, no phenotype specific DH sites could be identified within the panel of human melanoma cell lines tested. In addition, the transient expression assays with luciferase reporter gene vectors did not reveal differential promoter/enhancer activities in the analyzed calcyclin flanking sequences. The calcyclin promoter, contained in a *Sma*I-*Bam*HI fragment (-167 to +134) and included in DH site 0.0 (extending from -0.3 to +0.3), was active in all four human melanoma cell lines, which means that necessary transcription factors were present in the cell lines. The calcyclin promoter element includes a TATAA box, three GC boxes, and an enhancer-like structure [3]. When the calcyclin promoter element was extended upstream with 194 bp (-361/+134 *Xho*I-*Bam*HI), the luciferase activity was upregulated 2-3 fold in all cell lines. A further extension upstream of the calcyclin promoter element had no significant effect on the luciferase activity.

The enhancer activity present in the *Xho*I-*Sma*I region (-361/-167) coincides with the DH site 0.0. Differences in DH sites arise from nucleosome clearance of chromatin and changes in nucleosome spacing. Because of the intensity of DH site 0.0, it probably spans both promoter and enhancer. The lower accessibility of DH site 0.0 in the poorly metastasizing cell lines 1F6 and 530 may reflect normal nucleosome arrangements and related gene expression levels, while the chromatin structure of this region may be more disturbed in MV3 and BLM due to the reported rearrangements [14] and, consequently, more accessible to the relevant transcription factors. Although we cannot exclude the existence of more distant enhancer sequences, the present results support the view that the enhanced calcyclin gene expression in the metastasizing melanoma cell lines can be

ascribed to genomic rearrangements causing both gene amplification and increase in accessibility of the enhancer/ promoter sequences.

REFERENCES

1. Calabretta, B., Battini, R., Kaczmarek, L., De Riel, J.K. and Baserga, R. (1986) J. Biol. Chem. 261, 12628-12632.
2. Ferrari, S., Calabretta, B., deRiel, J.K., Battini, R., Ghezzo, F., Lauret, E., Griffin, C., Emanuel, B.S., Gurrieri, F. and Baserga, R. (1987) J. Biol. Chem. 262, 8325-8332.
3. Talavera, A. and Basilico, C. (1977) J. Cell. Physiol. 92, 425-436.
4. Rittling, S.R., Brooks, K.M, Cristofalo, V.J. and Baserga, R. (1986) Biochem. Biophys. Res. Commun. 261, 12628-12632.
5. Hirschhorn, R.R., Aller, D., Yuan, Z., Gibson, C.W. and Baserga, R. (1984) Proc Natl Acad Sci USA 81, 6004-6008.
6. Filipek, A., Heizmann, C.W. and Kuznicki, J. (1990) FEBS Lett. 264, 263-266.
7. Weterman, M.A.J., van Muijen, G.N.P., Bloemers, H.P.J. and Ruiter, D.J. (1993) Cancer Res. 53, 6061-6066.
8. Guo, X.J., Chambers, A.F., Parfett, C.L., Waterhouse, P., Murphy, L.C., Reid, R.E., Craig, A.M., Edwards, D.R. and Denhardt, D.T. (1990) Cell Growth Differ. 1, 333-338.
9. Murphy, L.C., Murphy, L.J., Tsuyuki, D., Duckworth, M.L. and Shiu, R.P. (1988) J. Biol. Chem. 263, 2397-2401.
10. Watanabe, M., Ando, Y., Tokumitsu, H. and Hidaka, H. (1993) Biochem. Biophys. Res. Commun. 196, 1376-1382.
11. Minami, H., Tokumitsu, H., Mizutani, A., Watanabe, Y., Watanabe, M. and Hidaka, H. (1992) FEBS Lett. 305, 217-219.
12. Zeng, F.Y., Gerke, V. and Gabius, H.J. (1993) Int. J. Biochem. 25, 1019-1027.
13. Gabius, H.J., Bardosi, A., Gabius, S, Hellman, K.P., Karas, M. and Kratzin, H. (1989) Biochem Biophys Res Commun 163, 506-512.
14. Weterman, M.A.J., Stoopen, G.M., van Muijen, G.N.P., Kuznicki, J., Ruiter, D.J. and Bloemers, H.P.J. (1992) Cancer Res. 52, 1291-1296.
15. van Muijen, G.N., Cornelissen, L.M., Jansen, C.F., Figdor, C.G., Johnson, J.P., Brocker, E.B. and Ruiter, D.J. (1991) Clin. Exp. Metastasis 9, 259-272.
16. van Muijen, G.N.P., Jansen, C.P.J., Cornelissen, L.M.A.H., Smeets, D.F.C.M., Beck, J.L.M. and Ruiter, D.J. (1991) Int. J. Cancer 48, 85-91.
17. Auffray, C. and Rougeon, F. (1980) Eur. J. Biochem. 107, 303-314.
18. McMaster, G.K. and Carmichael, G.G. (1977) Proc. Natl. Acad. Sci. USA 74, 4835-4838.
19. Church, G.M. and Gilbert, W. (1984) Proc. Natl. Acad. Sci. USA 81, 1991-1995.
20. Dirks, R.P.H., Jansen, H.J., Onnekink, C., de Jonge, R.J.A. and Bloemers, H.P.J. (1993) Eur. J. Biochem. 216, 487-495.
21. Lustig, L. and Denduchis, B. (1993) Medicina-Buenos. Aires. 53, 357-363.
22. Edlund, T., Walker, M.D., Barr, P.J. and Rulter, W.J. (1985) Science 230, 912-916.
23. Ferrari, S., Calabretta, B., Selleri, L., Ceccherelli, G., Torelli, G. and Torelli, U. (1988) Leukemia 2, 160S-166S.
24. Kuznicki, J., Kordowska, J., Puzianowska, M. and Wozniewicz, B.M. (1992) Exp. Cell Res. 200, 425-430.
25. Wood, L., Carter, D., Mills, M., Hatzenbuhler, N. and Vogeli, G. (1991) J. Invest. Dermatol. 96, 383-387.
26. Okazaki, K., Niki, I., Iino, S., Kobayashi, S. and Hidaka, H. (1994) J. Biol. Chem. 269, 6149-6152.
27. Timmons, P.M., Chan, C.T.J., Rigby, P.W.J. and Poirier, F. (1993) J. Cell Sci. 104, 187-196.
28. Creutz, C.E. (1992) Science 258, 924-931.