31. Southern, E. M. J. molec. Biol. 98, 503-517 (1975).
32. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. J. molec. Biol. 113, 237-252 (1977).
33. Shaw, A. R. & Ziff, E. B. Cell 22, 905-916 (1980).
34. Akusjarvi, G. & Persson, H. Nature 292, 420-426 (1981).
35. Thomas, G. P. & Mathews, M. B. Cell 22, 523-533 (1980).
36. Treisman, R., Green, M. R. & Maniatis, T. Proc. natn. Acad. Sci. U.S.A. 80, 7428-7432 (1983).
37. Miller, A. M. & Nasmyth, K. A. Nature 312, 247-251 (1984).
38. Chow, K.-C. & Pearson, G. D. Proc. natn. Acad. Sci. U.S.A. 82, 2247-2251 (1985).
39. Frost, E. & Williams, J. J. Virol. 91, 39-50 (1978).
40. Kimura, G. & Dulbecco, R. Virology 49, 394-403 (1972).
41. Harlow, E., Crawford, L. V., Pim, D. C. & Williamson, N. M. J. Virol. 39, 861-869 (1981).
42. Lewis, E. D., Fu, X.-Y. & Manley, J. L. ICN-UCLA Symp. molec. Biol. Dev. 19, 351-360 (1984).
43. Buchman, G., Burnett, L. & Berg, P. in DNA Tumor Viruses 2nd edn (ed. Tooze, J.) 799-841 (Cold Spring Harbor Laboratory, New York, 1981).
44. Tooze, J. (ed.) DNA Tumor Viruses 2nd edn (Cold Spring Harbor Laboratory, New York, 1981).
45. Gluzman, Y. Cell 23, 175-182 (1981).
46. Berk, A. J. & Sharp, P. A. Proc. natn. Acad. Sci. U.S.A. 75, 1274-1278 (1978).
47. Maxam, A. M. & Gilbert, W. Meth. Enzym. 65, 499-561 (1980).
48. Southern, E. M. J. molec. Biol. 98, 503-517 (1975).
49. Manos, M. M. & Gluzman, Y. Molec. cell. Biol. 4, 1125-1133 (1984).
50. Lewis, E. D. and Manley, J. L. Molec. cell. Biol. (in the press).

Chromosomal localization of a unique gene by non-autoradiographic *in situ* hybridization

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During the past few years, several methods have been developed for the detection of specific nucleic acid sequences by *in situ* hybridization using non-radioactive labels such as fluorochromes, cytochemically detectable enzymes and electron-dense markers[1–4]. These methods are preferable to autoradiography in terms of speed of performance and topological resolution. Their limited sensitivity, however, has so far restricted their use to the detection of repeated sequences. Here we report single gene detection with a procedure using 2-acetylaminofluorene (AAF)-modified probes, immunoperoxidase cytochemistry and reflection-contrast microscopy. We confirmed the autoradiographic data on the localization of the human thyroglobulin (*Tg*) gene to the distal end of the long arm of chromosome 8 (ref. 5). A mixture of cosmid cHT2-derived subclones of the 3′ part of the *Tg* gene, 22.3 kilobase pairs (kbp) in total, was used as a hybridization probe. This procedure can be used to map other unique sequences, if genomic clones are available from which clones with an appropriate amount of inserts can be isolated.

In non-autoradiographic procedures, labels are either coupled directly to the probe[6,7] or introduced via specific antibodies after hybridization with haptenized probes[2,8]. The procedure described here is an example of the latter, in which the nucleic acids are chemically modified with *N*-acetoxy-2-acetylaminofluorene (*N*-AcO-AAF). This compound reacts mainly at the C-8 position of guanine, yielding *N*-(guanin-8-yl)-AAF adducts[9]. Using anti-AAF antibodies and a fluorochrome-conjugated second-layer antiserum, we could localize high- and middle-repetitive sequences on mammalian metaphase chromosomes[10]. Recent developments in the visualization of *in situ* hybridization results have prompted us to investigate whether this procedure could be used for the detection of unique sequences. The *Tg* gene was used, because the gene is well characterized[11,12] and recently localized by more conventional methods such as Southern blot analysis of human-rodent somatic cell hybrids with human-specific chromosomal losses and *in situ* hybridization with titrated probes[5].

To establish a non-autoradiographic assignment of the *Tg* gene, hybridizations *in situ* with AAF-labelled probes were performed. A mixture of seven single-copy subclones derived from cosmid cHT2 was used to eliminate highly repeated sequences in genomic cosmid DNA. This cosmid contains 45 kbp, 38 kbp of which comprise the 3′ end of the *Tg* gene[11]. The subclones cover a total of 22.3 kbp, including 3 of the 5 exons in this region (0.55 kbp in total), ~20 kbp of intronic DNA and 2 kbp of 3′-flanking sequences[12] (Fig. 1). The DNA of these recombinant plasmids was sonicated to a single-stranded (ss) length of ~400 bases and modified with *N*-AcO-AAF to a substitution degree of about 5%[10]. The probe mixture was hybridized to metaphase spreads of cultured human lymphocytes and detected by means of an indirect immunoperoxidase reaction.

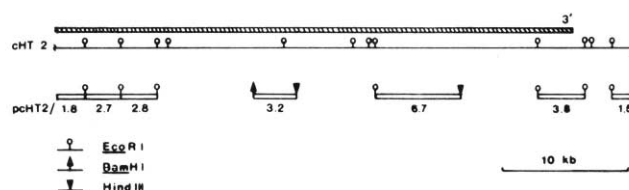

Fig. 1 Single-copy probes of the human thyroglobulin gene used for *in situ* hybridization (results from ref. 12). The upper bar shows the position of the 3′ end of the *Tg* gene with respect to cosmid cHT2 (line). The complete restriction maps for *Eco*RI, *Bam*HI and *Hin*dIII used for subcloning in pAT 153 are also indicated. The single-copy subclones are depicted as pcHT2/ followed by the length of their insert (lower bar).

The diaminobenzidine (DAB) precipitates, deposited at the chromosomal sites of hybridization, were observed with reflection-contrast microscopy[13,14]. This type of microscopy allows the visualization of reflection, rather than absorption of incident light by the dye precipitate. The light is reflected because of a critical difference in the refractive indices of the stained structure and the embedding medium, a phenomenon termed anomalous dispersion[15]. Because the DAB precipitate does not dissolve or recrystallize, these preparations are permanent and can be evaluated under the microscope several times without loss of detail. In addition to the specific hybridization signal, a low level of overall chromosome staining can be observed. This eliminates the need for a supplementary total DNA counterstain (Fig. 2). The applied combination of *in situ* hybridization and visualization also produces a banding pattern in the chromosomes resembling R-bands[16]. Although the banding is not as detailed as can be obtained with, for example, a routine G-banding procedure[17], the chromosomes can be karyotyped. Direct microscope evaluation of the chromosomes is possible but a higher contrast is obtained on photographs taken from the metaphases. Standard banding protocols, when tested, had the disadvantage that specific hybridization signals could no longer be distinguished from the other staining results.

The exact localization of the *Tg* gene was determined by analysing the distribution of DAB reflection signals from 40 metaphase spreads of a representative slide. The scoring was performed from photographs of randomly selected metaphases by two observers who were unaware of the gene involved. Different types of signals can be seen (Fig. 2), as dust particles or other contaminants present in the incubation solutions or immersion oil can cause reflection of the incident light. These background signals can be readily distinguished from DAB reflection by a few criteria, such as a different colour (blue instead of yellow), a halo effect, or a difference in focus plane between the signal and the chromosomes. Of the total of 99 chromosomal sites labelled, 36 (36%) were located at the end of the long arm of chromosome 8 and the remainder were randomly distributed over the chromosomes (Fig. 3). More illustrative, 45% of the individual metaphases showed one or more chromatids of chromosome 8 containing a signal at the q24 band. In several instances, all four chromatids of a single metaphase spread were positive (Fig. 2*a*). The fact that not every possible specific hybridization site reacts, can at least partly be

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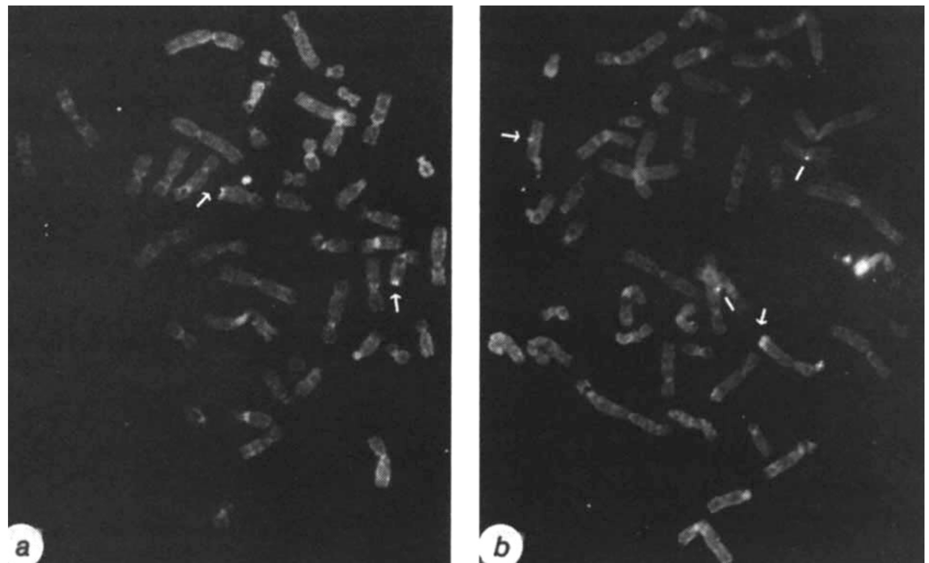

Fig. 2 Non-autoradiographic *in situ* hybridization of single-copy human *Tg* gene probes. Two examples of metaphases, which show in addition to the specific signals a weak overall DNA staining and an R-banding pattern, are shown. *a*, Each of the four chromatids is labelled at the end of the long arm of chromosome 8 (arrows). *b*, Both chromatids of one chromosome 8 are positive. In the latter metaphase, a DAB signal, which was also scored (Fig. 3), is indicated as well. A few background signals showing halo effects are marked by bars. Original magnification ×1,000.

Methods. A mixture of seven recombinant plasmids, derived from the 3′-end *Tg* cosmid clone cHT2 (see Fig. 1), was used as a probe. These plasmids, containing 22.3 kbp of unique fragments of the gene in total, were modified with AAF and hybridized to human metaphase preparations. The hybridized probes were detected by an indirect immunoperoxidase/diaminobenzidine reaction in combination with reflection-contrast microscopy. Chromosome preparations of cultured human peripheral blood lymphocytes were made according to conventional methods. Amounts of 1 μg DNA of each probe were pooled, sonicated to a ss length of ~400 bases, and labelled with *N*-acetoxy-2-acetylaminofluorene (*N*-AcO-AAF) to a substitution degree of about 5% (ref. 10). After purification the nucleic acids were dissolved at a concentration of 15 ng μl^{-1} in the hybridization mixture, containing salmon sperm DNA (750 ng μl^{-1}), formamide (50%, v/v) and 2×SSC (0.3 M NaCl, 30 mM Na-citrate, *p*H 7.0). Five μl of this mixture were applied per microscopic slide and hybridized for 18 h at 37 °C. The slides were denatured for 5 min in 0.07 M NaOH in 70% ethanol and treated for 15 min at 37 °C with proteinase K (10 μg per 100 ml 20 mM Tris-HCl, *p*H 7.5, 2 mM $CaCl_2$) prior to the hybridization. Immunocytochemical detection occurred through subsequent incubation with a rabbit anti-AAF serum (diluted 1:500 in phosphate-buffered saline (PBS) containing 2% normal serum and 0.05% Tween 20; SERVA), and a peroxidase-conjugated goat anti-rabbit serum (DAKO, diluted 1:50 in the same buffer) for 1 h at room temperature each. The slides were washed 3 times for 10 min in PBS between and after the incubations. The preparations were then stained with 0.01% 3,3′-diaminobenzidine tetrahydrochloride (Merck) and 0.01% H_2O_2 in 0.05 M Tris-HCl, *p*H 7.6 for 20 min at room temperature in the dark. After rinsing with PBS (3 times for 10 min), the preparations were dehydrated, air-dried and visualized by reflection-contrast microscopy[14].

explained by loss of chromosomal DNA. We have found that under optimal conditions this loss still equals ~30% of the DNA initially present (Raap *et al.*, in preparation). In control experiments, which included both unrelated AAF-modified probes and unrelated primary antibodies on hybridized slides, no significant labelling was observed.

This 'statistical localization' is typical of autoradiographical chromosomal mapping of single-copy genes[18,19]. The reported assignment of the *Tg* gene to the 8q24 band *in situ*[5], obtained by the use of a similar set of probes, required the observation of a significant number of silver grains in 50 spreads. However, those results could only be evaluated after exposure times varying from 2 to 3 weeks, whereas our slides can be viewed the next day. Another disadvantage of autoradiography is the location of most of the silver grains near the hybridized probe, rather than on the chromosome itself, because of the thickness of the emulsion.

In its present form the technique allows for the first time the non-autoradiographic detection of single-copy genomic sequences. In terms of slide preparation before hybridization, two elements are significant: the addition of alcohol to the denaturation mixture, which is important for the preservation of DNA sequences[10], and the application of proteinase K in enhancing accessibility for the antibodies[20]. The use of a set of unique recombinant plasmids as a hybridization probe and reflection-contrast microscopy may further account for the sensitivity obtained. This method of visualizing DAB precipitates favourably compares with absorption microscopy[14]; DAB signals on these preparations (both the banding pattern and the hybridization signals), for example, are not visible with the latter method.

Other unique genes can be localized by the strategy of using flanking sequences in combination with coding fragments. The necessary subcloning of single-copy segments from large genomic clones is not a disadvantage and usually required for gene characterization. The precise lower detection limit of the

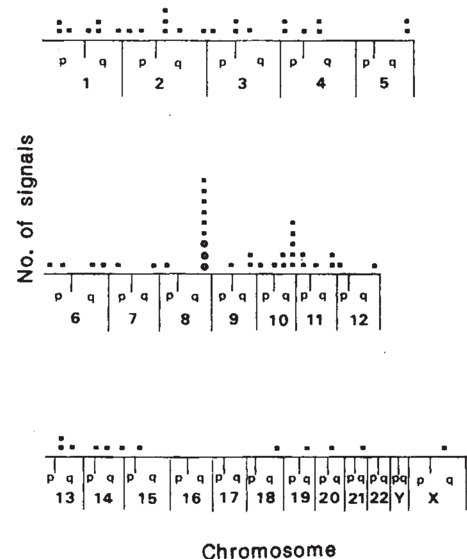

Fig. 3 Distribution of DAB reflection signals over metaphase chromosomes. Human chromosome preparations were hybridized with the AAF-labelled *Tg* probe mixture, visualized by means of the immunoperoxidase/reflection-contrast combination described in Fig. 2 legend and 40 randomly selected metaphases were analysed. The chromosomes were karyotyped by the R-banding pattern, which is provided by the procedure itself. The DAB reflection signals were scored and are marked as filled squares (■) above the schematic representation of the corresponding chromosome. Ten signals are indicated as filled circles (●). Thirty-six chromatids of chromosome 8 exhibited a signal at the distal part of the long arm (8q24 band), representing 36% of all labelled sites (99 in total) throughout the chromosome complement. This unambiguously confirms earlier autoradiographic data[5] on the localization of the *Tg* gene.

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procedure has not yet been determined, although it may be lower than the 22.3-kbp genomic DNA used here. Furthermore, the sensitivity of the method can still be improved at various levels of the procedure, for example, by optimalization of the hybridization efficiency. (The application of pure ssRNA, obtained by *in vitro* asymmetrical transcription of recombinants with SP6 RNA polymerase[21] has been reported to result in an eightfold increase of the *in situ* hybridization signals[22].) Improvements also seem possible at the level of immunocytochemical detection. The use of the streptavidin biotinylated-peroxidase system in combination with AAF-modified probes has proved to be advantageous over an indirect peroxidase reaction[23]. With such refinements, the localization of smaller single-copy fragments should be feasible. The speed of detection and the high resolution of the technique should greatly favour its applicability for the localization of structural genes or breakpoints in chromosomal rearrangements. In particular, the determination of the orientation of loci within a chromosome[24] would benefit from a non-autoradiographic approach.

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1. Rudkin, G. T. & Stollar, B. D. *Nature* **265**, 472-473 (1977).
2. Langer-Safer, P. R., Levine, M. & Ward, D. C. *Proc. natn. Acad. Sci. U.S.A.* **79**, 4381-4385 (1982).
3. Bauman, J. G. J., Van der Ploeg, M. & Van Duijn, P. in *Investigative Microtechniques in Medicine and Biology* (eds Chayen, J. & Bitensky, L.) 41-87 (Dekker, New York, 1984).
4. Manuelidis, L., Langer-Safer, P. R. & Ward, D. C. *J. Cell Biol.* **95**, 619-625 (1982).
5. Baas, F. *et al. Hum. Genet.* **69**, 138-143 (1985).
6. Bauman, J. G. J., Wiegant, J., Borst, P. & Van Duijn, P. *Expl Cell Res.* **138**, 485-490 (1980).
7. Renz, M. *EMBO J.* **2**, 817-822 (1983).
8. Shroyer, K. R. & Nakane, P. K. *J. Cell Biol.* **97**, 377a (1983).
9. Kriek, E., Miller, J. A., Juhl, U. & Miller, E. C. *Biochemistry* **6**, 177-182 (1967).
10. Landegent, J. E., Jansen in de Wal, N., Baan, R. A., Hoeijmakers, J. H. J. & Van der Ploeg, M. *Expl Cell Res.* **153**, 61-72 (1984).
11. Van Ommen, G.-J. B. *et al. Nucleic Acids Res.* **11**, 2273-2285 (1983).
12. Baas, F., Bikker, H., Van Ommen, G.-J. B. & De Vijlder, J. J. M. *Hum. Genet.* **67**, 301-305 (1984).
13. Ploem, J. S. in *Mononuclear Phagocytes in Immunity, Infection and Pathology* (ed. Van Furth, R.) 405-421 (Blackwell, Oxford, 1975).
14. Landegent, J. E., Jansen in de Wal, N., Ploem, J. S. & Van der Ploeg, M. *J. Histochem. Cytochem.* (in the press).
15. Van der Ploeg, M. & Van Duijn, P. *Histochemistry* **62**, 227-232 (1979).
16. Dutrillaux, B. & Lejeune, J. *C.r. hebd. Séanc. Acad. Sci., Paris* **272**, 2638-2640 (1971).
17. Sumner, A. T., Evans, H. J. & Buckland, R. A. *Nature new Biol.* **232**, 31-32 (1971).
18. Gerhard, D. S., Kawasaki, E. S., Bancroft, F. C. & Szabo, P. *Proc. natn. Acad. Sci. U.S.A.* **78**, 3755-3759 (1981).
19. Harper, M. E., Ullrich, A. & Saunders, G. F. *Proc. natn. Acad. Sci. U.S.A.* **78**, 4458-4460 (1981).
20. Van Prooijen-Knegt, A. C. *et al. Expl Cell Res.* **141**, 397-407 (1982).
21. Melton, D. A. *et al. Nucleic Acids Res.* **12**, 7035-7056 (1984).
22. Cox, K. H., Deleon, D. V., Angerer, L. M. & Angerer, R. C. *Devl Biol.* **101**, 485-502 (1984).
23. Heyting, C., Kroes, W. G. M., Kriek, E., Meyer, I. & Slater, R. *Acta histochem.* (in the press).
24. Morton, C. C. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 2816-2820 (1984).

Characterization of 64-, 123- and 182-base-pair exons in the mouse α2(IV) collagen gene

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Genes encoding types I, II and III collagens (fibrillar collagens) contain many discrete-size exons, most of them 54 base pairs (bp) long, in addition to the 45-, 99-, 108- and 162-bp exons[1–6]. It has been suggested[2,6] that these collagen genes evolved from an ancestral coding unit of 54 bp. Type IV collagen is a specific component of basement membranes and contains two genetically distinct polypeptides, the α1(IV) and α2(IV) chains[7–9]. It differs from the types I–III collagens in that it contains interruptions in the Gly-X-Y repeat sequence[10–12] and does not form ordered fibrillar structures[7,13]. We have isolated complementary DNA and genomic clones for the mouse α2(IV) collagen chain and here characterize 64-, 123- and 182-bp exons in the Gly-X-Y coding domain of the gene. The data suggest that the α2(IV) collagen gene may have evolved differently from those encoding the fibrillar collagens.

From a mouse parietal endoderm (PE) cDNA library[14], we isolated a cDNA clone which by messenger RNA hybrid selection and *in vitro* translation was shown to be specific for one of the type IV collagen chains. In Northern analysis (Fig. 1) this cDNA, PE69 (430 bp), hybridizes to a 6.4-kilobase (kb) RNA, distinct from the major 6.8-kb and minor 6.2-kb RNA species which hybridize with a mouse cDNA probe specific for α1(IV) collagen chain (refs 14, 15 and M.K. *et al.*, in preparation). The nucleotide sequence of PE69 cDNA was determined and an amino-acid sequence of 143 residues was derived from it which contained Gly-X-Y repeats with three interruptions (Fig. 2). This sequence overlapped and was nearly identical with the partial protein sequence available from mouse and human α2(IV) collagen chains[16]. From these results we conclude that the mouse PE69 cDNA, reported here, is specific for the α2(IV) collagen chain. At present, the exact position of this sequence within the 1,740-amino-acid[17] α2(IV) collagen chain is not known. However, the mouse protein sequence (Fig. 2) represents the N-terminus of pepsin fragment P2, of relative molecular mass 70,000, which is located near the N-terminal end of the

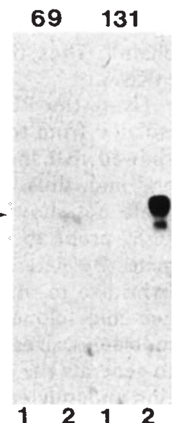

Fig. 1 Northern hybridization. Total cellular RNA (10 μg) of mouse embryo parietal endoderm cells (lane 2) and F9 embryonal carcinoma cells (lane 1) were run on an agarose - formaldehyde gel and transferred onto a nitrocellulose filter. One-half of the filter was hybridized with the PE69 cDNA probe and the other half with an α1(IV) collagen-chain-specific cDNA probe, PE131 (refs 14, 15). These probes hybridized to a 6.4-kb RNA (arrowhead) and a major 6.8-kb and minor 6.2-kb RNA, respectively, visible only in lane 2. As reported previously[14], mouse parietal endoderm cells synthesize 100-fold more type IV collagen than do the F9 embryonal carcinoma cells. Size markers were mouse ribosomal RNAs[21] visualized in the gel by ethidium bromide staining.

Methods. A cDNA expression library was constructed from total poly(A)$^+$RNA of 13.5-day mouse embryo parietal endoderm (PE) cells by the double-linker method[22]. The double-stranded cDNA, with an *Eco*RI linker at the end corresponding to the 5′ end of the mRNA and a *Sal*I linker corresponding to the 3′ end of the mRNA, was digested to completion with both *Eco*RI and *Sal*I and size fractionated on a Sepharose 4B column. cDNAs ~500 bp and larger were then ligated to *Eco*RI/*Sal*I double-cut vector pUC8 (ref. 23). Transformation of *Escherichia coli* strain DH-1 with recombinant plasmids was carried out as described previously[24]. The bacteria were plated onto 82-mm nitrocellulose filters overlaid on ampicillin (50 μg ml^{-1}) plates to give 1,000-2,000 colonies per filter. For long-term storage at −70 °C, colonies were replica plated onto nitrocellulose filters and replicas were grown on 5% glycerol plates[25]. Duplicate filters of the PE cDNA expression library (~25,000 colonies) were screened with antibodies as described previously[22] using a mixture of antisera against mouse laminin and entactin[26] and mouse PYS-2 cells[27], a teratocarcinoma-derived PE cell line that produces large amounts of type IV collagen. After this 'shotgun' screening nine signal clones were selected for further analysis by Northern hybridization, mRNA hybrid selection and *in vitro* translation[14]. One clone (pPE69) was found to hybrid select mRNA for a polypeptide of 168,000 relative molecular mass, similar in size to one of the *in vitro* synthesized chains of mouse type IV collagen[9]. The mouse library was constructed in the hope that cDNA clones expressing sequences for basement membrane components or other abundant PE proteins could be identified directly by immunological screening. Indeed, in separate experiments, one cDNA clone expressing a fragment of mouse laminin B2 chain was isolated by this technique[28]. However, isolation of the pPE69 clone from the PE cDNA expression library by antibody screening was only fortuitous. Sequence analysis of PE69 cDNA revealed, quite surprisingly, that the DNA strand corresponding to mRNA is in the wrong orientation for expression in the pUC8 vector (Fig. 2).

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