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LOSS OF CHROMOSOME 9 IN TISSUE SECTIONS OF TRANSITIONAL CELL CARCINOMAS AS DETECTED BY INTERPHASE CYTOGENETICS. A COMPARISON WITH RFLP ANALYSIS

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SUMMARY

Interphase cytogenetics by *in situ* hybridization (ISH) using a panel of centromere-associated DNA probes for chromosomes 1, 7, 9, 10, 11, 16, 17, and 18 was performed on 5 µm thick frozen tissue sections of transitional cell carcinomas (TCCs) of the urinary bladder. By this approach, chromosome ploidy, numerical chromosome aberrations, imbalance between chromosomes, and heterogeneity of aberrations within individual tumours were determined. In 15 of 24 TCCs, loss or underrepresentation of chromosome 9, compared with the ISH copy numbers of at least five other chromosomes, was demonstrated. Independently, RFLP analysis were performed on the same cases to detect loss of heterozygosity (LOH) of chromosome loci 9q34, 11p15, 16q22–24, 17p13, and 18q21. LOH was found in 9 of 19 informative cases for chromosome locus 9q34. Comparison of the ISH and RFLP results showed no correlation between numerical aberration and LOH for the loci on chromosomes 11, 16, 17, and 18. However, numerical loss of chromosome 9 was found in 89 per cent (eight of nine cases) with LOH for 9q34. Conversely, LOH at 9q34 was observed in only 67 per cent (eight of 12 cases) with underrepresentation of chromosome 9. Moreover, in 60 per cent of the non-informative cases (three of five cases), underrepresentation for chromosome 9 was observed. These results indicate that the heterochromatin probe for chromosome 9 can be reliably used in TCC tissue sections for the detection of chromosomal loss. In aneuploid TCCs, this DNA probe can be used for the detection of chromosomal underrepresentation only in combination with other centromere-associated DNA probes.

KEY WORDS—bladder cancer; chromosome aberrations; *in situ* hybridization; RFLP analysis

INTRODUCTION

Transitional cell carcinoma (TCC) is the most common form of urinary bladder cancer, comprising a heterogeneous group with markedly different neoplastic features.¹ Approximately two-thirds of patients with TCC sooner or later present recurrences, of which 10–25 per cent will be of higher grade or stage.² Currently, the most important predictors of the behaviour of TCCs are histological stage and grade. The DNA ploidy, as determined by flow cytometry (FCM), may have additional value in prediction of the biological behaviour of the tumour.³ For example, FCM has revealed that non-invasive (pTa) TCCs are predominantly DNA-diploid, while invasive behaviour of these malignancies is highly correlated with DNA aneuploidy or tetraploidy.^{4–6} Cytogenetic analysis by karyotyping of TCCs has revealed several specific chromosomal changes, such as -9 or $-9q-$.^{7–11}

Comparison of tumour DNA and constitutional DNA for polymorphic markers by restriction fragment length polymorphism (RFLP) analysis allows the detection of loss of heterozygosity (LOH) at specific chromosomal loci.¹² RFLP analyses have shown that allelic loss

on 9q, 11p, 16q, 17p, and 18q is involved in various human cancers, including urinary bladder cancers.^{13–18} The RFLP probe for 9q (9q34 locus), in particular, has been shown to be indicative for changes in chromosome 9 in bladder cancer. The existence of two allelic loci has been postulated, one in each of both chromosomal arms.¹⁹ Recently, the deleted regions on chromosome 9 in bladder cancer were better defined.²⁰ It has been suggested that 9p LOH may be associated with a more aggressive biological behaviour or early disease progression.²⁰

Interphase cytogenetics by *in situ* hybridization (ISH) using centromere-associated DNA probes is applied to both single cell suspensions and sections of frozen tissues or formalin-fixed, paraffin-embedded tissues.^{21–30} In this study, interphase cytogenetic analysis was performed to detect numerical chromosomal aberrations in standard 5 µm thick frozen tissue sections. Selection of the probes was made on basis of the literature, indicating specific loss for chromosomes 9, 10, and 11, or gain for chromosomes 1 and 7.^{31,32} In addition, centromeric probes for chromosomes 16, 17, and 18 were selected, of which no numerical changes have been reported. We used several RFLP probes on the p- or q-arms of these chromosomes to detect LOH in the DNA extracted from serial sections of the same samples.

Although the DNA probes for ISH recognize repetitive sequences in the centromeric regions of

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chromosomes and the RFLP probes were used to detect LOH on the p- or q-arms, we focused the comparison on the following questions: (a) is there a correlation between underrepresentation of certain chromosomes, as detected by ISH, and LOH of markers on that same chromosome? (b) Can heterogeneity of chromosome aberrations be detected in TCC by means of ISH and how does such heterogeneity influence the RFLP results? (c) To what extent do both techniques overlap and thus reveal the same information?

MATERIALS AND METHODS

Sample selection and preparation for ISH

The tissue samples were selected for their tumour cell content, which was at least 60–70 per cent. Classification of histological stage and grade (I–III) was performed using paraffin sections. For this study, 24 frozen tissue samples of TCC were selected, on which both ISH and RFLP techniques could be performed. For ISH, 5 μ m sections were mounted on organosilane-coated slides, air-dried, and stored at room temperature. For RFLP analysis, 20 μ m sections were collected and stored at -20°C . Also, peripheral blood lymphocytes (PBLs) were collected from the same patients for RFLP analysis.

Pretreatment of frozen sections

Prior to the ISH procedure, the sections were fixed in 70 per cent ethanol/1 per cent formaldehyde for 20 min at -20°C . After subsequent washing in phosphate-buffered saline (PBS)/0.05 per cent Tween-20, and water for 5 min at room temperature, the slides were incubated for 15 min at 37°C in 100 μ g pepsin per ml 0.01 N HCl. Then the slides were rinsed in five dip washes of water and five dip washes of PBS, and dehydrated in an ascending alcohol series. After air-drying, the sections were post-fixed in 1 per cent formaldehyde/PBS for 10 min at room temperature.

DNA probes for ISH

The centromere-associated DNA probes pUC1.77,³³ p7t.1,³⁴ pHUR98,³⁵ pHUR195,³⁵ p10.1,³⁶ pLC11A,³⁷ p17H8,³⁸ and L1.84³⁹ were used for the detection of target sequences on chromosomes 1, 7, 9, 16, 10, 11, 17, and 18, respectively. The probes were labelled by nick-translation with biotin-11-dATP, according to the supplier's labelling kit instructions (Boehringer, Mannheim, Germany).

In situ hybridization

The DNA probes described above were hybridized to the (tumour-) cell preparations at a probe concentration of 1 ng/ μ l hybridization mixture, containing 60 per cent formamide–2 x standard sodium citrate (SSC)–10 per cent dextran sulphate, pH 7.0. Under these stringent conditions, 15 μ l of the hybridization mixture was applied to the slides under a coverslip (18 \times 18 mm).

Denaturation of probe and target DNA was performed simultaneously by heating the slides in a moist chamber to 70°C for 3 min. After hybridization for 2–16 h at 37°C , the coverslips were removed by immersing the slides in 2 \times SSC, pH 7.0 at 42°C . Post-hybridization washing steps were performed twice in 60 per cent formamide–2 \times SSC, pH 7.0 for 5 min and twice in 2 \times SSC, pH 7.0 for 5 min at 42°C .

Immunocytochemical detection of the hybridized DNA probes was performed as previously described^{24,25} with mouse anti-biotin (DAKO A/S, Glostrup, Denmark), biotin-labelled horse anti-mouse IgG (Vector, Burlingame, CA, U.S.A.), and a final incubation with the avidin–biotin-labelled peroxidase complex (Vectastain Elite ABC Kit, Vector). All immunocytochemical steps were performed for 30 min at 37°C . The DNA probe was visualized with 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma), 0.65 per cent imidazole (Marck, Darmstadt, Germany), 0.015 per cent H_2O_2 (Merck) in PBS for 3 min at pH 7.8, and the signal was amplified with CuSO_4 (0.5 per cent in 0.9 per cent NaCl) for 3 min. The slides were counterstained with haematoxylin and mounted in Permount (Fisher Scientific, New Jersey, U.S.A.).

Evaluation of ISH results

The reproducibility and validity of a protocol to detect and evaluate chromosome copy numbers by ISH in 5 μ m routinely processed tissue sections have been described elsewhere.²⁵

A disadvantage of the use of tissue sections is that due to truncation of a significant number of nuclei, the number of ISH signals per nucleus will be underestimated compared with the actual copy number of the target chromosome.^{25–28} Therefore, we evaluated separately within each section the tumour area, normal tissue, and stromal cells, since no simple correction factors for truncation of nuclei are available so far. In diploid tumours, the true chromosome copy number is detectable in about 50 per cent of the nuclei, so that monosomy or trisomy can be determined conclusively. However, with increasing nuclear size, the true chromosome copy number will be increasingly underestimated. For this reason, hybridization with different DNA probes on the same tumour areas in parallel sections is necessary to study the imbalance of chromosome copy numbers and, as a result, the specific loss of chromosomes.

Statistical methods

The Kolmogorov–Smirnov test was used. Underrepresentation of a specific chromosome was seen as a shift to the left of the ISH signal distribution when compared with non-aberrant probe distributions. Conversely, gain of a specific chromosome was seen as a shift to the right. *P* values of 0.001 or less were considered significant.

DNA isolation and Southern blot analysis

RFLP analysis was performed as described previously.⁴⁰ Briefly, high molecular weight DNA (about

Table I—A summary of the RFLP and ISH results for the 24 transitional cell carcinomas. For ISH, the centromere-associated DNA probes for chromosomes 1, 7, 9, 10, 11, 16, 17, and 18 were used

Case No.	Grade/stage	RFLP analysis					ISH analysis	
		Chromosomal locus detected on					Chromosome ploidy	Numerical aberration(s)
		9q	11p	16q	17p	18q		
1	G1/pTa	+	+	+	+	+	Diploid	None
2	G1/pTa	+	+	+	+	NI	Diploid	-9, -17
3	G1/pTa	-	+	-	+	+	Diploid	-9
4	G1/pTa	+	+	+	+	NI	Diploid	-9, -17
5	G1-2/pTa	+	+	+	+	+	Diploid	-10
6	G1-2/pTa	-	-	+	+	+	Diploid	-9
7	G2/pTa	+	NI	+	-	-	Diploid	-9, -18
8	G2/pT1	-	+	+	+	NI	Diploid	-9
9	G2/pT1	-	-	+	+	NI	Diploid	+7, -9
10	G2/pT1	+	+	+	+	NI	Diploid	-18
11	G2/pT1	+	NI	+	NI	+	Diploid	None
12	G3/pT3	-	+	+	NI	nd	Diploid	-9
13	G3/pT3	NI	+	+	+	nd	Diploid	None
14	G2-3/pTa	NI	+	+	+	NI	Triploid	-9
15	G2/pT1	+	+	+	+	+	Triploid	+1, -9
16	G2/pT1	-	+	+	-	-	Tetraploid	+1, -9
17	G2/pT1	NI	-	+	+	-	Hexaploid	-9, -9
18	G1-2/pT1-2	+	NI	+	+	+	Tetraploid	None
19	G3/pT2	+	+	-	+	NI	Tetraploid	-18
20	G3/pT>2	NI	NI	+	+	NI	Tetraploid	-9, -17
21	G3/pT3	NI	NI	-	-	nd	Triploid	None
22	G1-2/pTa	-	+	+	-	nd	Tetraploid /diploid	+1, -9, -9 /+1, -9
23	G1-2/pTa	-	-	+	+	+	Tetraploid /diploid	None
24	G3/pT>2	-	+	+	-	+	Tetraploid /diploid	-9, -9, -17, -17 /-9, -17

NI=probe is not informative; nd=not done; +=no LOH; -=LOH.

25 µg) was isolated from frozen tissue samples of the 24 TCCs and from whole blood samples of the same patients as described.⁴¹ Ten µg of DNA was digested with TaqI of MspI and separated on a 0.8 per cent agarose gel and transferred to Hybond N+ (Amersham) according to the manufacturer's instructions. The ³²P dATP-labelled probes, EFD126 for 9q34,⁴² c-H-ras for 11p15,⁴³ PV962 and 79.2.23 for 16q22,^{44,45} 144D6 for 17p13,⁴⁶ and 15.65 for 18q21,⁴⁷ were hybridized overnight in 250 mM Na₂HPO₄, 1 mM EDTA, 1 per cent BSA, 7 per cent SDS, pH 7.2 at 65°C. Blots were then washed successively in 250 mM Na₂HPO₄, 1 mM EDTA, 1 per cent SDS, pH 7.2; in 125 mM Na₂HPO₄, 1 mM EDTA, 1 per cent SDS, pH 7.2; and in 50 mM Na₂HPO₄, 1 mM EDTA, 1 per cent SDS, pH 7.2.⁴⁰ Since tumour content was high enough, scanning of film was not necessary for interpretation. The RFLP analysis was performed blind and independently of the ISH analysis.

RESULTS

Table I summarizes the RFLP and ISH results for each of the 24 TCCs. These were ranged according to their chromosome ploidy as determined on basis of ISH.

Cases 1-13 were classified as chromosome diploid and cases 14-24 as aneuploid, of which cases 22-24 were also heterogeneous, i.e., mixed diploid and aneuploid.

Evaluation of the ISH reactions showed in 18 patients 27 times a loss or gain of ISH signals compared with the mean chromosome ploidy. A numerical loss of chromosome 9 was found in 15 cases (62.5 per cent). In 13 patients, we found 24 times LOH for one or more markers. The most frequent allelic loss was seen for the marker on chromosome 9q. Of 19 informative cases (79 per cent), we found LOH of the 9q34 locus in nine tumours (47 per cent).

Chromosome diploid tumours (cases 1-13)

Numerical chromosome aberrations were detected in 10 out of 13 diploid TCCs (Table I). In eight cases, a loss of chromosome 9 was observed. In two cases, an additional loss of chromosome 17 was detected. One case (case 4) demonstrated a loss of chromosome 10, whereas case 6 showed loss of chromosome 18, next to a loss of chromosome 9. Loss of chromosome 18 was also observed in case 9. Figure 1A shows the quantitative evaluation of the hybridization signals of case 1 for the centromere-associated DNA probes for chromosomes 1,

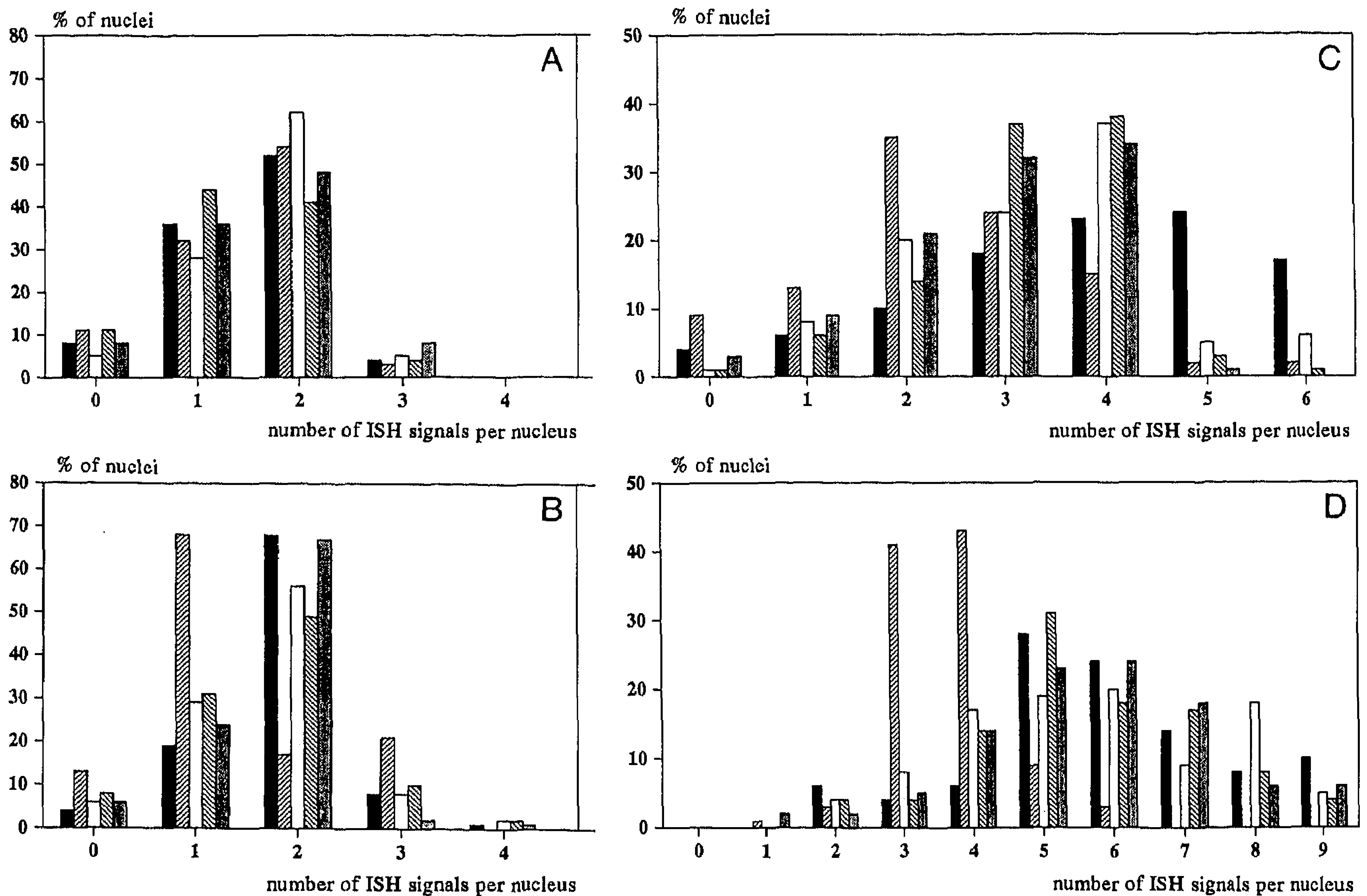


Fig. 1—Frequency distribution patterns of ISH signals obtained with different centromere-associated DNA probes for chromosome 1 (■), chromosome 9 (▨), chromosome 11 (□), chromosome 17 (▩), and chromosome 18 (▧) on frozen tissue sections of TCCs. (A) Case 1, representing a diploid tumour with no numerical aberrations. (B) Case 3, showing underrepresentation for chromosome 9. (C) Aneuploid tumour, case 16, demonstrating overrepresentation of chromosome centromere 1 and loss of the centromeric target of chromosome 9. (D) Case 17, representing a highly aneuploid tumour with loss of chromosome 9. The mean chromosome copy number is 6. Note the effect of truncation of nuclei on the distribution patterns of the chromosome copy numbers

9, 11, 17, and 18. In this particular case, the mean number of ISH signals for the chromosomal probes was two in about 45–50 per cent of the nuclei and one in 28–42 per cent of the nuclei. These percentages did not differ from those obtained in model studies.^{26,48} This case can therefore be classified as a chromosome diploid tumour. Furthermore, statistical analysis revealed no differences in the ISH signal distribution pattern of the chromosomes. Figure 1B represents case 3, in which a significant loss of chromosome 9 was detected (with all investigated chromosomes, $P < 0.001$), since 68 per cent of the nuclei contain only one ISH signal for the chromosome 9 probe. In about 55 per cent of the nuclei of this case, two ISH signals for the other chromosomes were detected. Figures 2A and 2B show tumour areas of this chromosome diploid case (case 3) in frozen tissue sections, hybridized with the probe for chromosome 11 (Fig. 2A), showing two ISH signals, and the probe for chromosome 9 (Fig. 2B), showing only a few cells containing two ISH signals per nucleus, indicating an evident monosomy for chromosome 9.

The RFLP results for cases 1 and 3 are shown in Figs 2C and 2D, on which Taq1-digested DNA is hybridized to a probe for 9q and 11p, respectively. Tumour DNA from case 3 showed LOH for the 9q probe (Fig. 2C),

whereas for the 11p probe no LOH was observed (Fig. 2D).

Chromosome aneuploid tumours (cases 14–24)

In 5 of 11 aneuploid cases, underrepresentation of chromosome 9, compared with the other chromosomes, was detected. In two cases (cases 15 and 16), overrepresentation of chromosome 1 was observed. An additional loss of chromosome 17, next to loss of chromosome 9, was detected in case 20. Case 19 showed loss of chromosome 18 as the only numerical aberration.

Figure 1C shows the frequency distribution of case 16 by evaluating the ISH signals in 200 nuclei in serial sections, showing a mean between 3.0 and 3.3 for the copy number of chromosomes 7, 11, 16, 17, and 18, and a modal copy number of four ISH signals per nucleus. For chromosome 1 the mean copy number was 3.9, and the modal copy number was five to six ISH signals per nucleus, representing a relative overrepresentation or gain of this chromosome (P values < 0.001). The mean and modal copy numbers for chromosome 9 were 2.4 and 2, respectively, demonstrating an underrepresentation or loss of this chromosome (P values < 0.001).

The frequency distribution of ISH signals for chromosomes 1, 9, 11, 17, and 18 of case 17 is shown in Fig. 1D.

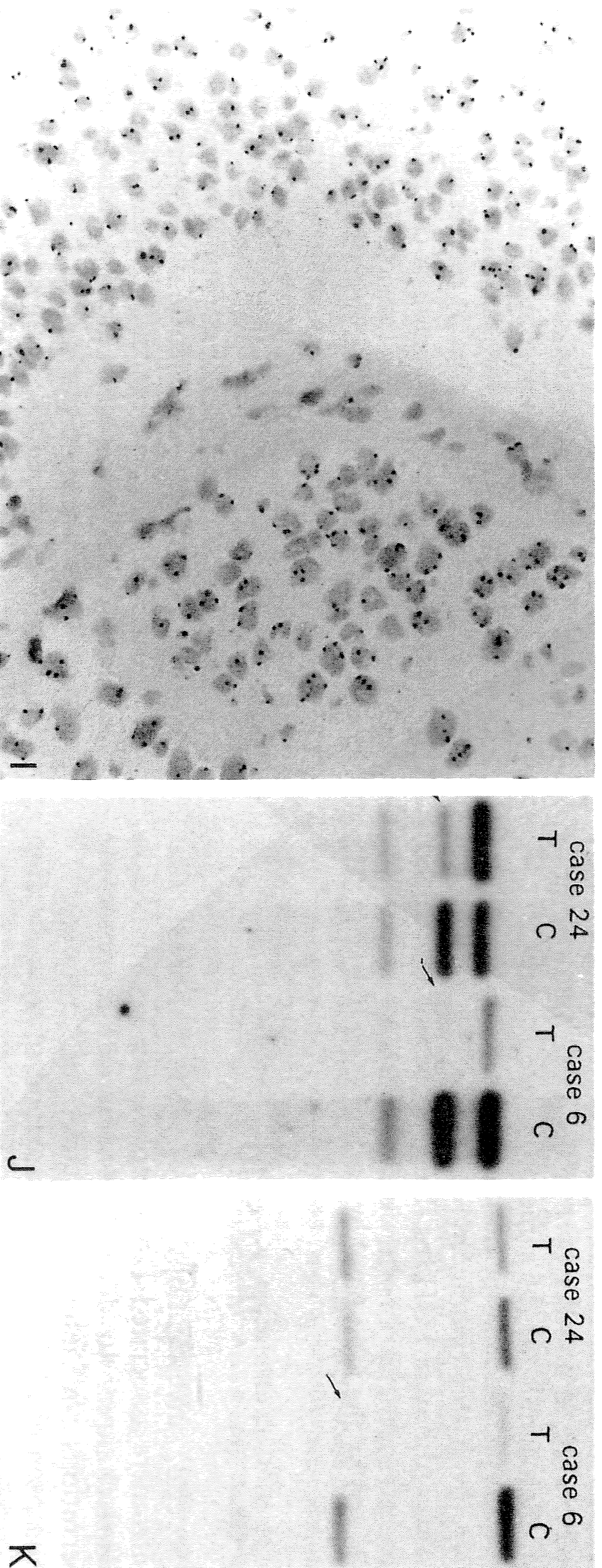
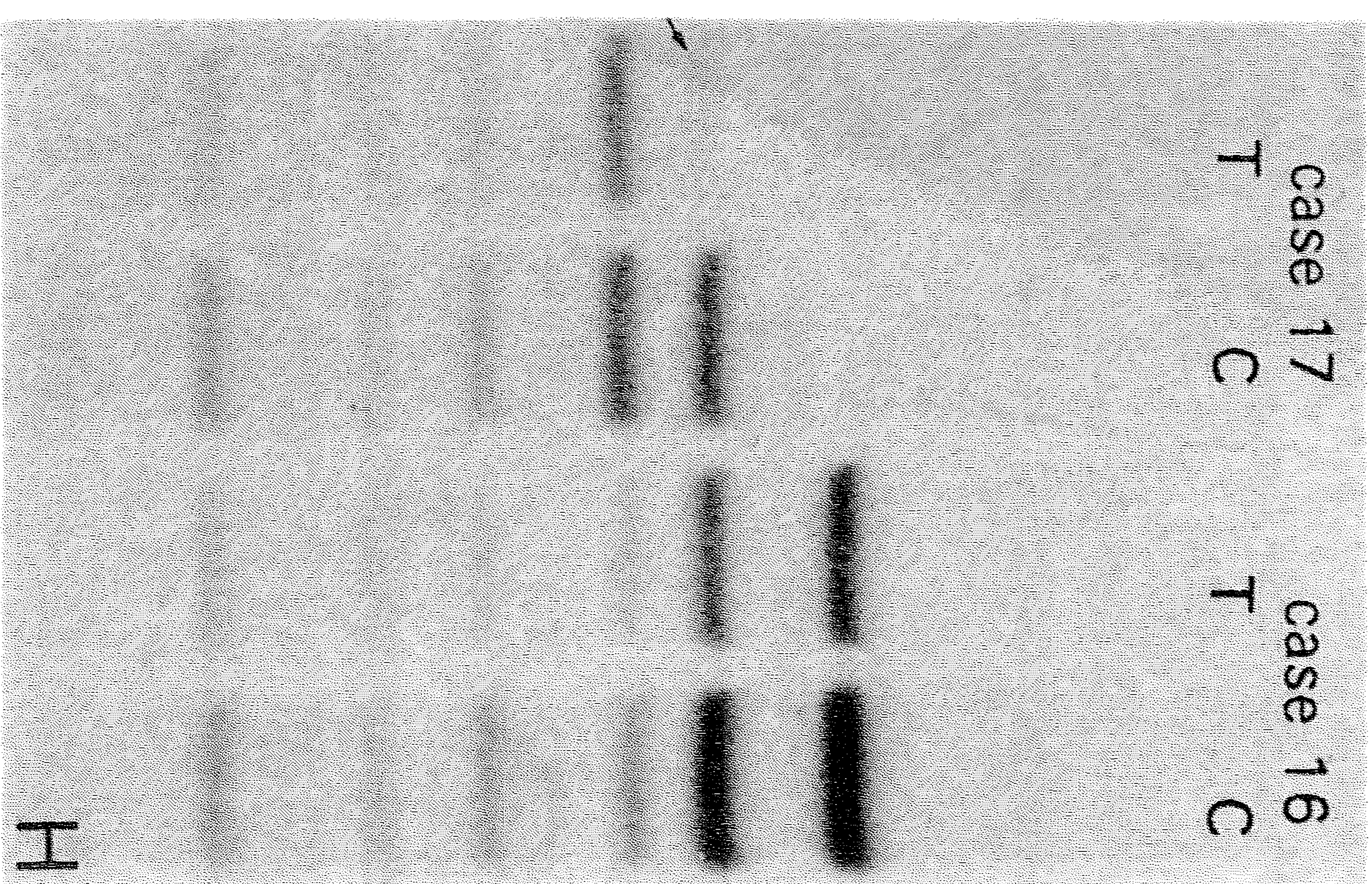
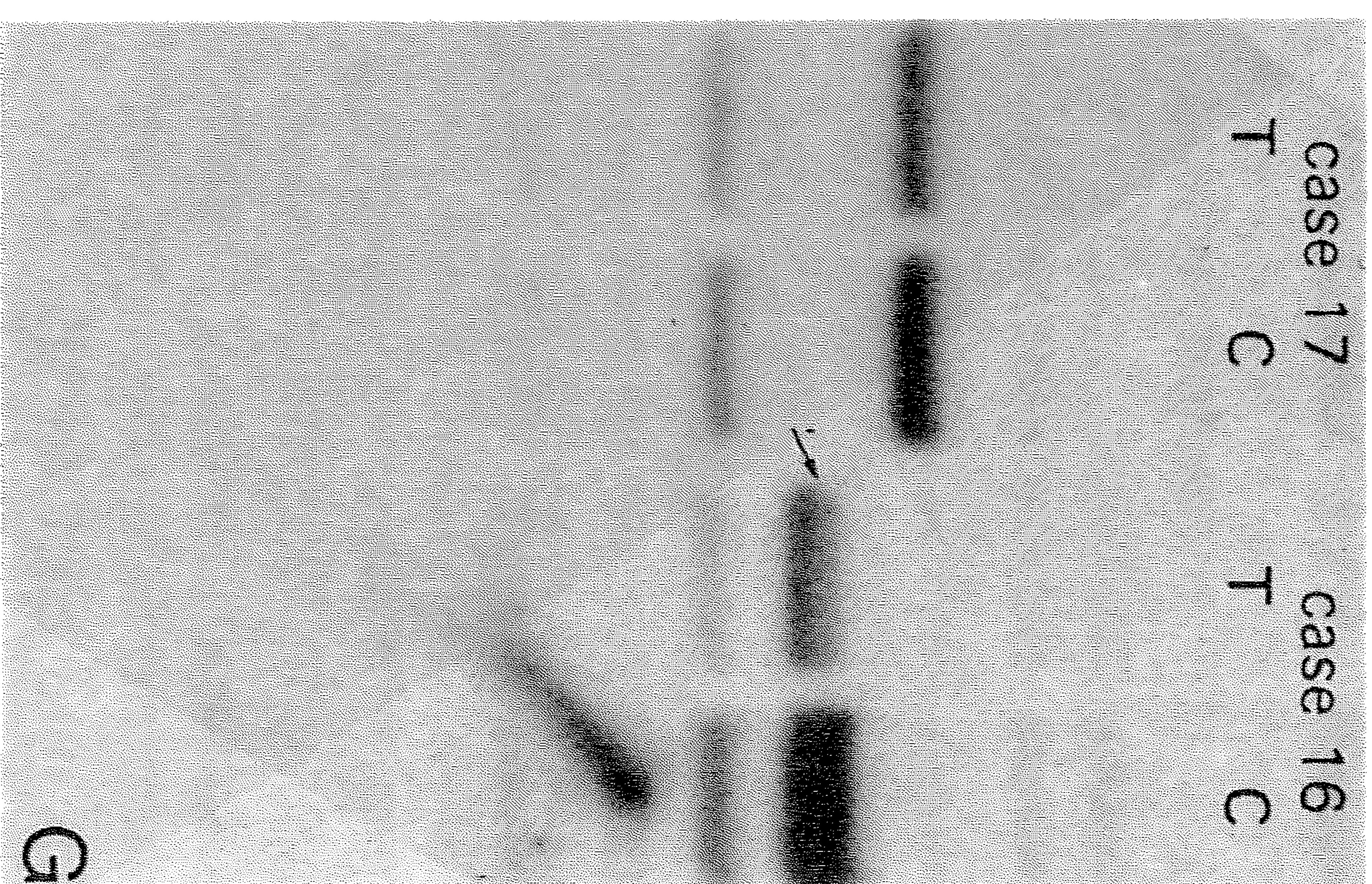
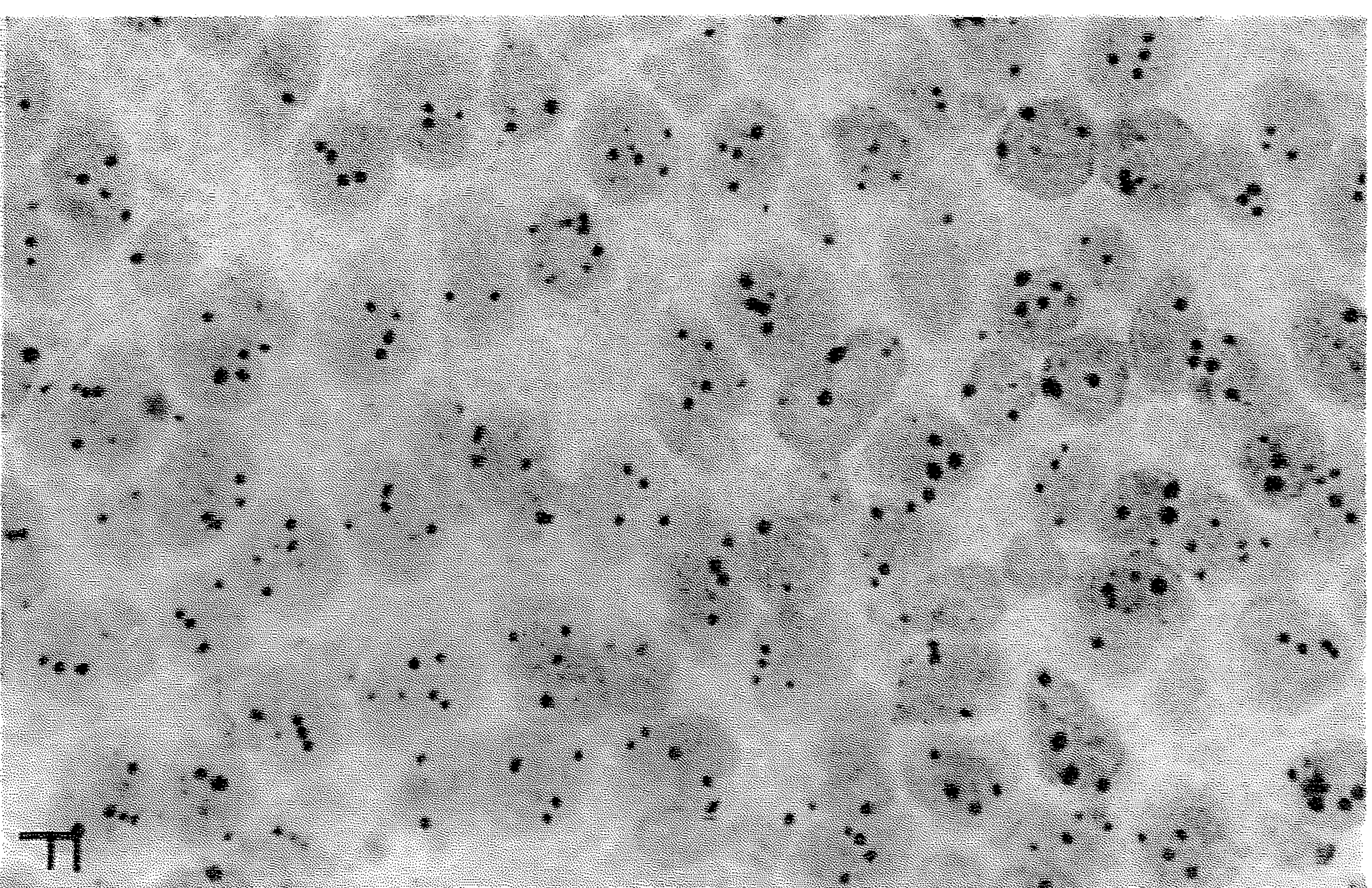
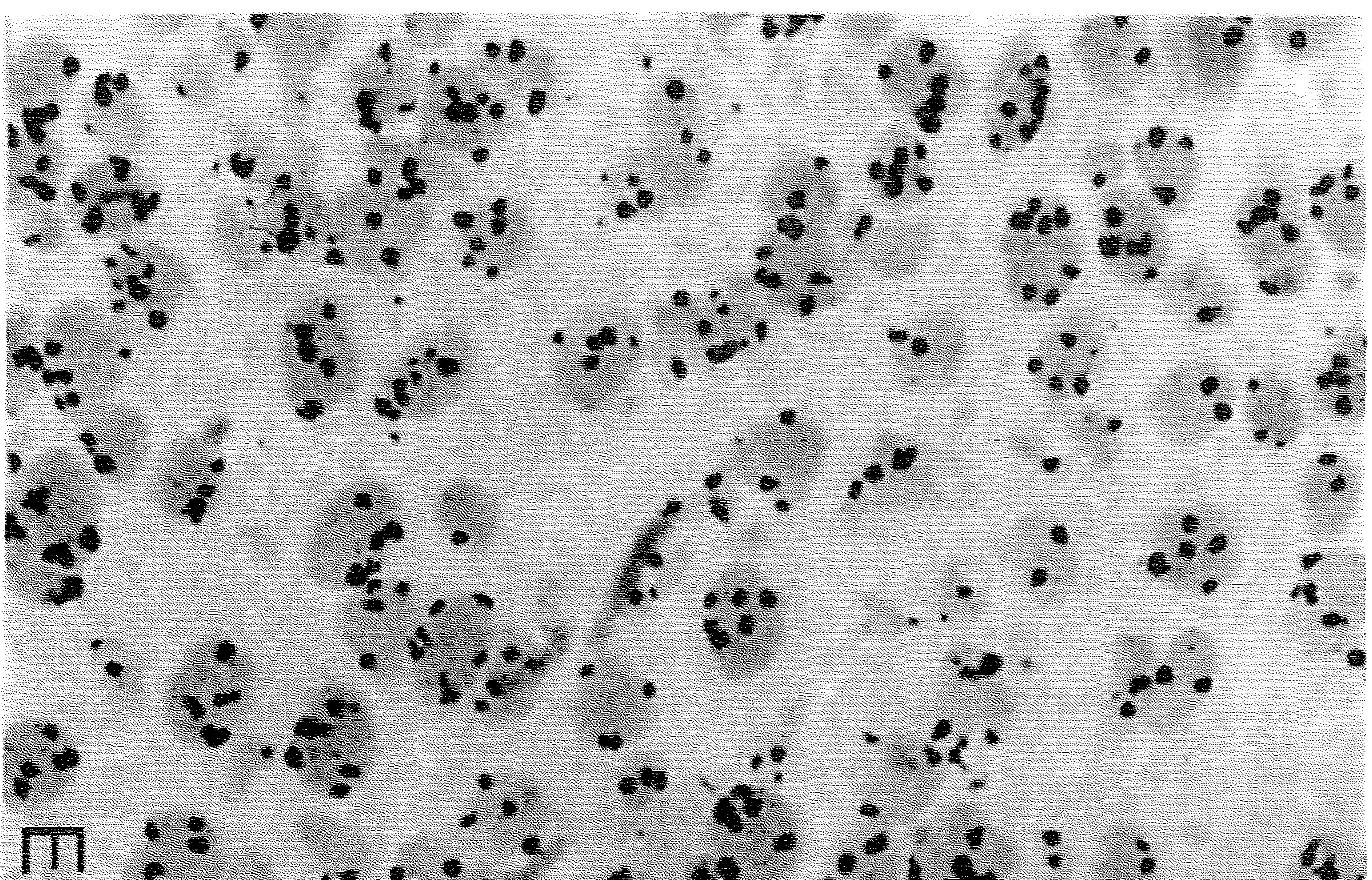
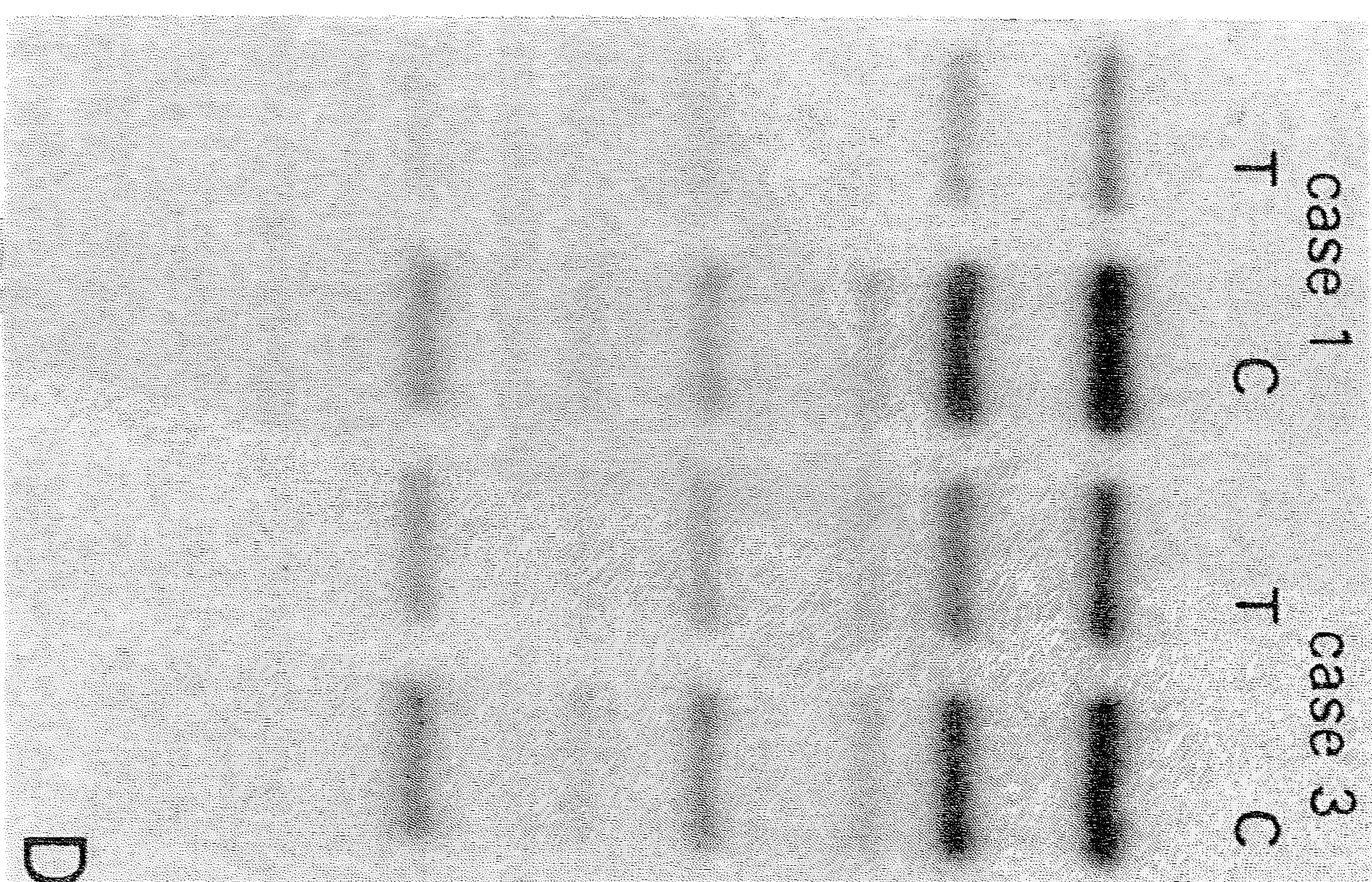
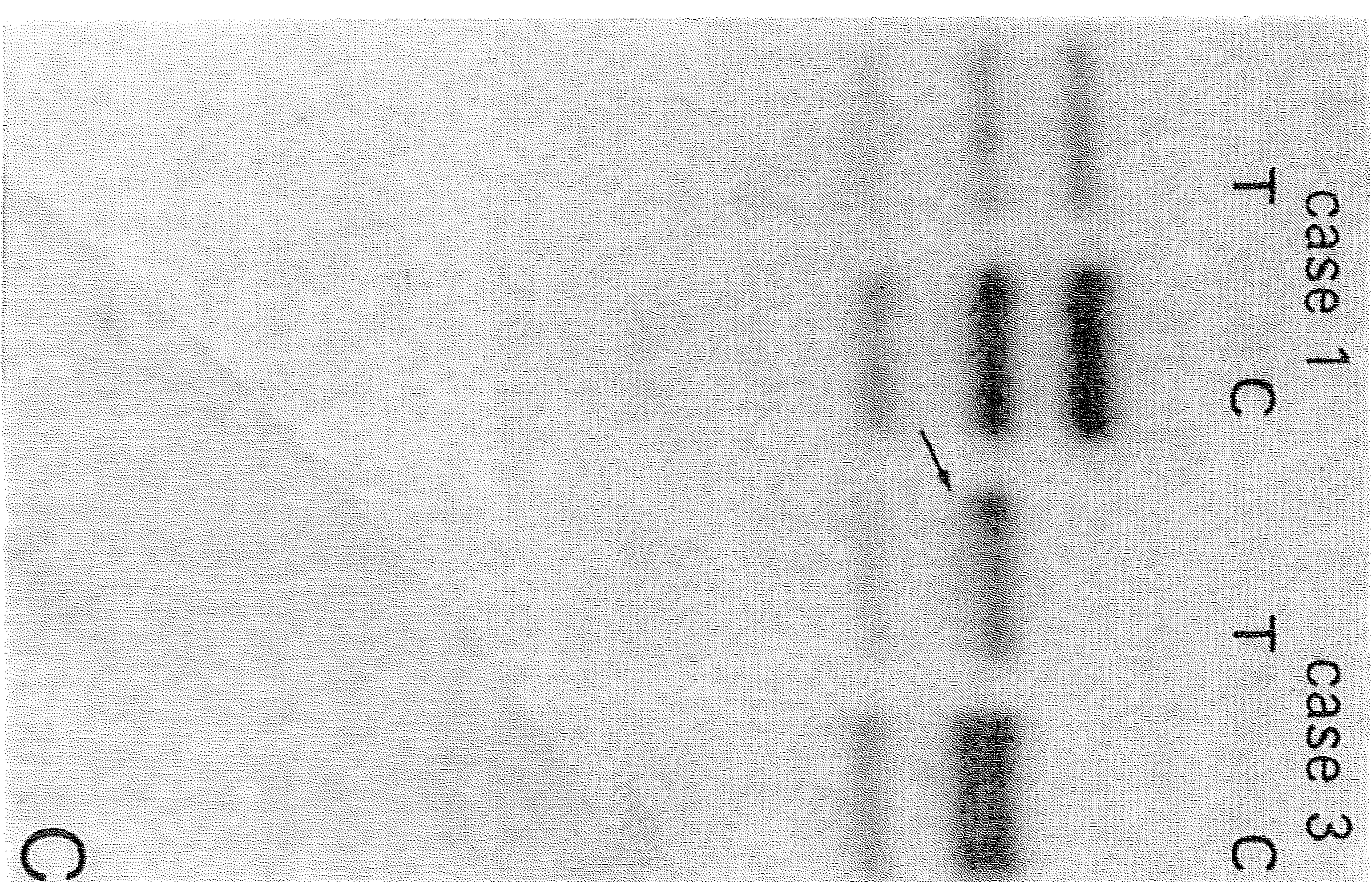
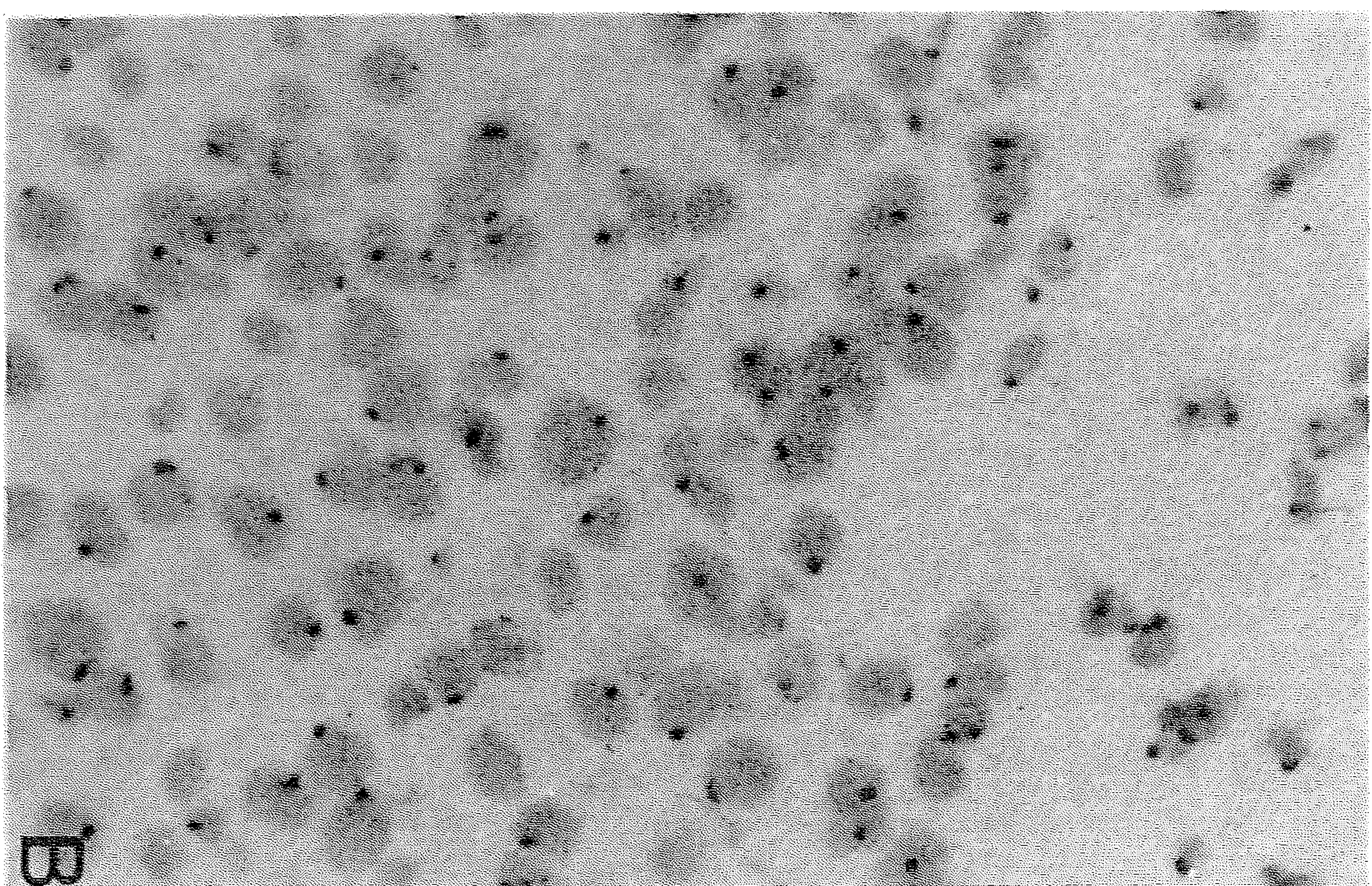
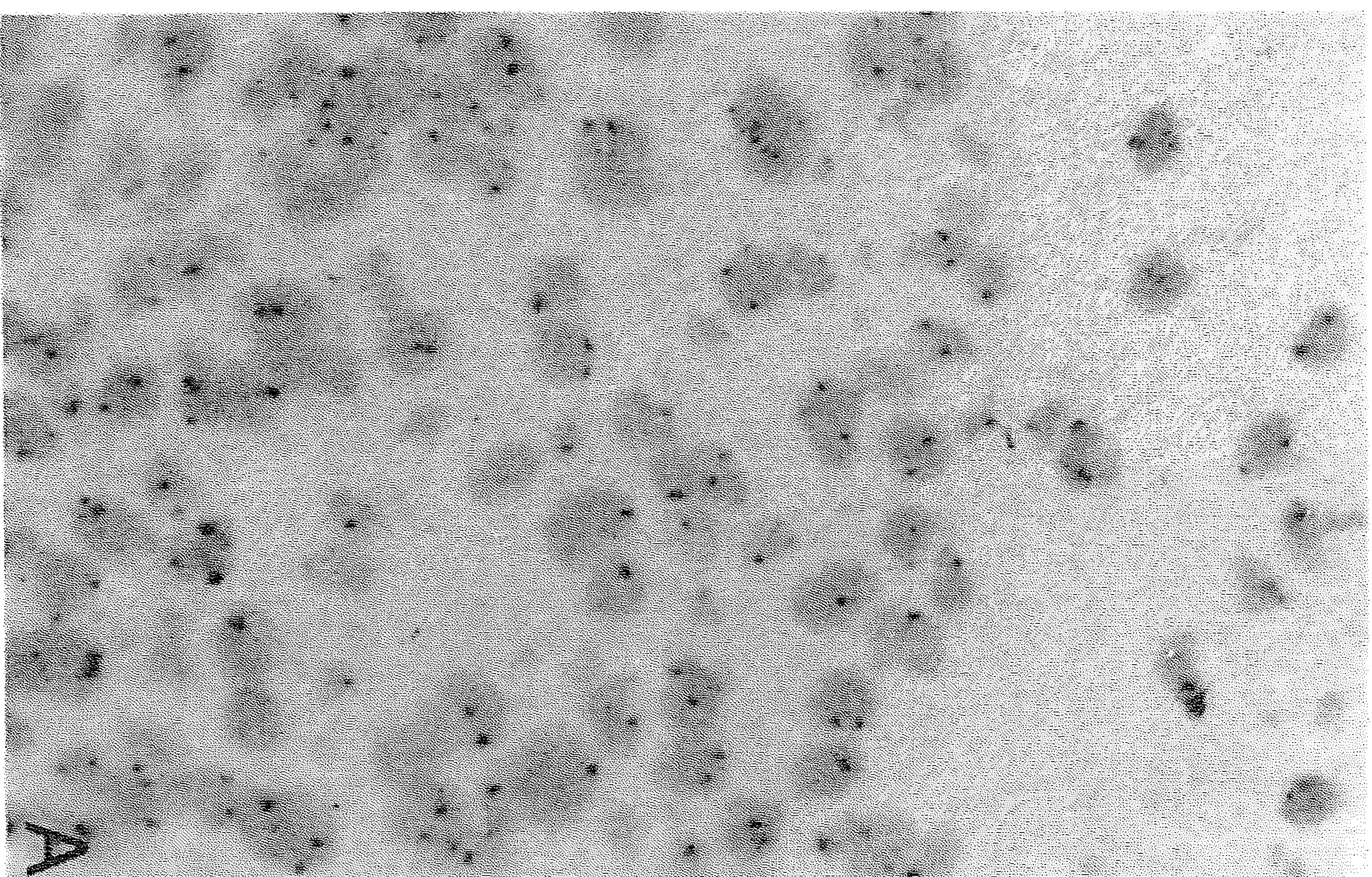


Fig. 2—DNA-ISH and Southern blot hybridizations of tumour (T) and constitutional (C) DNA of TCCs. A–D represent case 3. In serial 5 μ m sections, a maximum of two ISH signals for chromosome 11 (A) and a maximum of one ISH signal for chromosome 9 (B) are shown. Southern blot analyses show for case 1 retention (C: lane 1) and for case 3 LOH (C: lane 3) for the 9q34 probe. Both cases were heterozygous for the 11p probe (D: lanes 2 and 4, respectively) with no LOH (D: lanes 1 and 3). E–H represent case 17. DNA-ISH shows nuclei containing five to six ISH signals for chromosome 1 (E). The arrow indicates a nucleus with eight ISH signals. In F, the same tumour area in a serial section is hybridized with the probe for chromosome 9, showing a maximum of four ISH signals. Southern blot analyses for the 9q34 probe revealed that tumour DNA from case 16 showed LOH (G: lane 3), whereas case 17 retained the 9q34 locus (G: lane 2). For the 11p probe, both tumours were informative (H: lanes 2 and 4). Case 17 demonstrated LOH (H: lane 1) and case 16 no LOH for this locus (H: lane 3). I–K represent cases 6 and 24. In I, the heterogeneity of case 24 is demonstrated for the chromosome 1 probe. To the left, the nuclei contain mainly two ISH signals, while to the right, the nuclei contain three and four ISH signals. By RFLP analysis, case 24 (J: lane 1) showed LOH for 9q34, but not LOH for the 11p marker (K: lane 1). Case 6 showed LOH for both 9q34 (J: lane 3) and 11p (K: lane 3)



The mean copy number for chromosomes 1, 11, 17, and 18 was between 5.6 and 5.9, and there was a modal copy number of six ISH signals per nucleus. For chromosome 9 the mean copy number was 3.6, with a modal copy number of 4, representing a loss of two copies of this chromosome (P values < 0.001). Figures 2E and 2F represent part of the tumour of case 17, where the nuclei contain five to six ISH signals, with a maximum ISH copy number of 9, for chromosome 1 (Fig. 2E), whereas for chromosome 9 the nuclei contain three to four ISH signals, with a maximum ISH copy number of 6 (Fig. 2F).

Southern blots of tumour and constitutional DNA from cases 16 and 17 are shown in Figures 2G and 2H, hybridized to the 9q and 11p probe, respectively. For the 9q probe, case 16 demonstrates LOH, while case 17 is not informative (Fig. 2G). For the 11p probe, case 16 retains the 11p allele, whereas case 17 reveals LOH (Fig. 2H).

Evaluation of ISH signals for the three aneuploid heterogeneous cases (cases 22–24) revealed extensive chromosome heterogeneity, both in the range of ISH signals for the eight different DNA probes in the individual tumour cells and between different tumour areas in the same case of TCC. We have therefore classified these three cases as heterogeneous. In two cases (cases 22 and 24), loss of the heterochromatin region of chromosome 9 was detected after screening of serial sections with the eight centromere-associated DNA probes. In the tetraploid tumour areas, we observed an ISH copy number of chromosome 9 of maximum two indicated as an apparent loss of two copies of chromosome 9 (Table 1). Figure 2I demonstrates the tumour heterogeneity of ISH signals in case 24, in which one part of the TCC contains three or four ISH signals for the chromosome 1 probe, while in another part of the TCC mainly one or two ISH signals for the same probe are found.

Figures 2J and 2K demonstrate the RFLP results of DNA from this tumour hybridized to the 9q and 11p probes. Although LOH for the 9q locus was concluded, the heterogeneity of the tumour DNA is possibly reflected by the faint band in the blot (arrow in Fig. 2J).

DISCUSSION

Several investigations have demonstrated that centromere-associated DNA probes can be applied in ISH techniques to determine numerical chromosome aberrations in interphase nuclei of tumours.^{21–30} Thompson *et al.*⁴⁹ described a method of hybridization to sections more than 20 μ m thick, to overcome difficulties in the analysis of gene or chromosome copy number due to truncation of nuclei. Since these analyses have to be performed using laser-scanning confocal microscopy, this approach is not routinely applicable.

Our study shows the application of ISH using a panel of DNA probes to standard 5 μ m thick frozen tissue sections of TCCs, in order to detect numerical chromosome aberrations and chromosomal imbalances. The most characteristic numerical aberration detected by ISH in the 24 TCCs was a loss of chromosome 9 in 15 of

24 cases (62.5 per cent; P values < 0.001), compared with the mean chromosome ploidy determined with the panel of eight centromere-associated DNA probes. These observations strongly confirm other published data,^{31,32} where the cytogenetic observations concerning loss of chromosome 9 in low-grade, low-stage TCCs were confirmed by the ISH approach. Moreover, the frequent occurrence of loss of chromosome 9 in early stages of TCC is in agreement with the observation of Tsai *et al.*,¹³ who showed that LOH of markers on chromosome 9q could be detected in 67 per cent of the informative cases. Comparison of the ISH results with RFLP analysis revealed that numerical loss of chromosome 9 was found in 89 per cent of the cases with LOH for 9q34. However, only 67 per cent of cases with loss of chromosome 9 as detected by ISH showed LOH for 9q34.

The process of tetraploidization is a generally accepted concept in tumour progression.¹² In the aneuploid TCCs, the apparent loss of two copies of chromosome 9 strongly indicates that the loss of one copy of this chromosome occurred before tetraploidization took place. This confirms our previous suggestion that loss of chromosome 9 is a primary or early event in carcinogenesis of the urinary bladder³¹ and that it is conserved during the process of tumour progression and invasion. The loss of chromosome 9 as detected by ISH is most probably not limited to a small part of 9q. Because this DNA target is situated in the heterochromatin on 9q, close to the centromere, only a complete loss of this heterochromatin region would result in a complete disappearance of the chromosome 9 signal. Deletion of either 9q or 9p would preserve part of this target sequence, resulting in positive but less intense or less extended ISH signals. In our RFLP analysis we used one polymorphic marker located at 9q34. This may explain our observation that in four cases (2, 4, 7, and 15) there was an ISH loss of chromosome 9, but no LOH was observed with RFLP analysis. These cases should involve complex chromosomal rearrangements, with loss of the heterochromatin region of chromosome 9 and retention of the 9q34 region. Translocation of part of the 9q-arm could be the most likely explanation.

For the other chromosomes studied, we found no correlation between LOH and centromeric loss. The biological meaning of these observations could be that in those cases where LOH was found but no loss was observed by ISH for that particular chromosome, an interstitial deletion, chromosomal rearrangement, or duplication of a part of that chromosome was involved.

Putative tumour suppressor genes involved in bladder carcinoma are located at the region between 9p12–13 and 9q34.^{20,51,52} The loss of the putative tumour suppressor gene on chromosome 9 also comprises, in most cases, the heterochromatin region on 9q. ISH with a centromere 9 probe can therefore be used to monitor this loss in pathological specimens. On the other hand, loss of tumour suppressor genes on chromosomes 11p and 17p, which is frequent in bladder tumours, is not associated with the loss of any entire chromosome or with partial loss including the centromere. Screening for loss of these tumour suppressor genes by ISH should be done using specific cosmid probes located near the

relevant locus. In this respect, the strategies of Matsumura *et al.*⁵³ demonstrated that LOH of 17p in breast tumours correlated with loss of ISH signals of a 17p cosmid probe. No correlation was found with the centromeric probe. Similarly in our studies on bladder tumours, no correlation was found with the copy number for the centromeric probe.

ISH analysis using tissue sections is a more specific approach than RFLP to karyotyping, since it is possible to evaluate results at the individual cell level, while Southern blot analysis provides a quantitative but integral overview of changes that dominate within the tumour. In our study, in three aneuploid cases in which LOH for the chromosome 9 locus was found, we detected a DNA diploid tumour area next to the DNA aneuploid area. An ISH approach can discriminate between these. Tissue microdissection and subsequent PCR-RFLP analysis will allow the examination of LOH of specific chromosomal regions.^{54,55} Different clones that might exist among neoplastic cells can then be determined and studied in greater detail.

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