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High-resolution chromosomal localization of human genes for amylase, proopiomelanocortin, somatostatin, and a DNA fragment (*D3S1*) by *in situ* hybridization

(gene mapping/prometaphase chromosomes/recombinant DNA probes)

BERNHARD U. ZABEL*, SUSAN L. NAYLOR*, ALAN Y. SAKAGUCHI*, GRAEME I. BELL†, AND THOMAS B. SHOWS*

*Department of Human Genetics, Roswell Park Memorial Institute, New York State Department of Health, Buffalo, NY 14263; and †Chiron Corporation, Emeryville, CA 94608

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ABSTRACT **The method of *in situ* hybridization has become a significant technique for specific-site chromosome mapping. We show that the resolution of *in situ* hybridization can be increased by hybridizing the probe to stretched prometaphase chromosomes with high-resolution banding obtained after 5-bromodeoxyuridine treatment of the cells and with a Hoechst 33258/Giemsa chromosome-staining method. Using this procedure, we assigned to specific chromosome sites three cloned genes and one DNA polymorphism: amylase gene (*AMY*) to 1p21; proopiomelanocortin gene (*POMC*) to 2p23, somatostatin gene (*SST*) to 3q28, and a single copy DNA segment (*D3S1*) to 3q12.**

Assignment of human genes to specific sites on chromosomes can be accomplished visually by the method of *in situ* hybridization. The technique, previously limited to detection of repetitive gene families, now allows identification of low- and single-copy genes. The increased sensitivity has been achieved by using nick-translated recombinant DNA probes that form networks with the high molecular weight polymer dextran sulfate (1–7) or by using cRNA probes of high specific activities made from genomic DNA recombinants (8–10). *In situ* hybridization has extended the precision of gene mapping and complements other mapping methods. We show here that the resolution of the method of *in situ* hybridization can be improved by hybridizing the probe to large prometaphase chromosomes that display a distinct banding pattern of up to 1,000 bands per haploid set of chromosomes. Longer chromosomes with a distinct banding pattern are essential for higher accuracy of scoring, which is necessary for the precise localization of a DNA probe. Most studies have relied on relatively compact metaphase chromosomes stained either before or after *in situ* hybridization; these usually display an indistinct banding pattern. We used bromodeoxyuridine (BrdUrd) as the cell-synchronizing agent and a reliable Hoechst 33258/Giemsa chromosome-staining method at the end of the *in situ* hybridization procedure. With these methods, we obtain a higher percentage of long prometaphase chromosomes with high-resolution G-banding than with the method using chromosomes from amethopterin-synchronized lymphocyte cultures (2). A technique using meiotic chromosome preparations to increase banding resolution has been described (6) but is generally not applicable on a routine basis.

Using these refined procedures with high-resolution G-banded prophase and prometaphase chromosomes, we have assigned to specific chromosome sites the three cloned genes for amylase (*AMY*), proopiomelanocortin (*POMC*), and somatostatin (*SST*) and an undefined DNA fragment (*D3S1*) that identifies a DNA polymorphism. Specific-site mapping of these loci will prove important because (*i*) amylase has been an extensively studied marker for family studies on chromosome 1 (11), (*ii*) proopiomelanocortin is a complex precursor for several peptides (corticotropin, β-lipotropin, β-endorphin, and melanotropin) that are mapped by the assignment of *POMC* (12), (*iii*) somatostatin is a hormone and possibly a neuropeptide whose exact mapping also localizes two polymorphic DNA sites on chromosome 3 (13), and (*iv*) *D3S1* is an example of the potentially large group of undefined DNA segments that detect DNA polymorphisms (14).

MATERIALS AND METHODS

Chromosome Preparation. A variation of the Dutrillaux and Viegas–Pequignot (15) method was used. Twelve drops of heparin-treated blood was cultured in 4 ml of chromosome medium 1A with phytohemagglutinin (GIBCO) for 72 hr at 37°C; 200 μg of BrdUrd per ml of medium (Sigma) was added, and after 16–17 hr the cells were washed twice with medium and incubated in fresh chromosome medium containing 10 μM thymidine (Sigma). Colcemid was not necessary to obtain metaphase cells by this method. After 6.5–7 hr, the cells were centrifuged (5 min at 200 × *g*) and resuspended in 6 ml of 0.075 M KCl (37°C) for 8 min of hypotonic treatment. The cells were fixed with methanol/glacial acetic acid, 3:1 (vol/vol), for 20 min, washed three times with fixative, and dropped on ice-cold slides. From the time of addition of BrdUrd, the cells and slides were protected from direct light exposure to avoid nicking the DNA.

The slides (1–14 days old) were treated (under a cover glass) with preboiled RNase A (100 μg/ml; Sigma) in 2× SSC (1× SSC = 0.15 M NaCl/0.015 M trisodium citrate, pH 7.0) for 60 min at 37°C, washed several times in 2× SSC, dehydrated in 70% and 95% ethanol, and air dried.

DNA Probes. Recombinant DNA probes for amylase, proopiomelanocortin, somatostatin, and *D3S1* were isolated from human DNA libraries and cloned in bacteriophage λ or plasmid pBR322 as reported (12, 14, 16). We used a Charon 4A clone with a 14.5-kilobase (kb) insert containing an *AMY* gene (λhamy1), a 2-kb *Eco*RI–*Bgl*II fragment of the human *POMC* gene subcloned into pBR322 (pHA3BgR2) (12), a 2.7-kb fragment of human DNA including the *SST* gene cloned in pBR322 (pgHS7-2.7) (16), and a 2.3-kb single copy DNA segment of chromosome 3, *D3S1*, cloned in Charon 16A (HS-3) (14).

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Abbreviations: *AMY*, amylase gene; *POMC*, proopiomelanocortin gene; *SST*, somatostatin gene; *D3S1*, single-copy DNA fragment; BrdUrd, 5-bromodeoxyuridine; kb, kilobase(s).

Radiolabeling of DNA Probes. Nick-translation was essentially as described (17). The 100-μl reaction mixture contained 1 μg of DNA, 6 μM of three labeled nucleotides ([^{3}H]dATP at 19.2 Ci/mmol, [^{3}H]dCTP at 45.3 Ci/mmol, and [^{3}H]TTP at 97.7 Ci/mmol, New England Nuclear; 1 Ci = 37 × GBq), and 60 μM of unlabeled dGTP in buffer (50 mM Tris·HCl, pH 7.8/5 mM $MgCl_2$/10 mM 2-mercaptoethanol/5 μg of bovine serum albumin per ml). The DNA was nicked with 10 pg of activated DNase I (Boehringer Mannheim) for 10 min at 15°C, and then 18 units of *Escherichia coli* polymerase I (Boehringer Mannheim) was added for about 3 hr until 1–2 × 10^7 cpm were incorporated; the reaction was stopped by adding 10 μl of 0.25 M EDTA (pH 8.0). The labeled DNA was separated from unincorporated nucleotides by centrifugation through a 1-ml Bio-Gel P-60 (BioRad) column made in a punctured Eppendorf tube (2 min at 200 × *g*). Approximately 70% of the total label incorporated into DNA was recovered. Fifty microliters of sonicated salmon sperm DNA (10 mg/ml) was added to the 200-μl eluate. The probe was hybridized in 50% deionized formamide/10% dextran sulfate/2× SSC 40 mM sodium phosphate/0.1% $NaDodSO_4$/1× Denhardt's solution (0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.2% bovine serum albumin (final pH 7.0–7.2).

***In Situ* Hybridization.** A modification of the method of Harper and Saunders (2) was used for hybridization. The chromosomes on the slides were denatured in 70% formamide/2× SSC at 70°C for 2 min, followed by quick dehydration sequentially in cold (−20°C) 70% and 95% ethanol and air dried. The probe mixture was heated at 70°C for 5 min, frozen in a dry-ice ethanol bath, and after thawing placed on slides under a siliconized coverslip (100 μl with 50–100 ng of DNA per slide). Incubation was carried out in a 2× SSC saturated environment in Petri dishes for 24 hr at 37°C. The slides were dipped in 50% formamide/2× SSC, pH 7.0, at 39–40°C to remove the coverslip, washed twice for 10 min in 50% formamide/2× SSC and three times for 10 min in 2× SSC—all at 39–40°C. Slides were then washed three times for 10 min in 2× SSC at room temperature, dehydrated in 70% and 95% ethanol, and air dried.

Autoradiography. Slides were dipped for 1 sec in NTB2 (Kodak)/H_2O 1:1 (vol/vol) (42°C). After being air dried in the dark for 2 hr, the slides were put in a light-tight box with Drierite for 1–2 wk at 4°C. Slides were developed in Kodak D-19, diluted 1:1 with water for 4 min at 15°C, fixed for 5 min in Kodak fixer, and rinsed in distilled water for 15 min followed by air drying for 24 hr.

Staining (G-Banding). A variation of the method of Wolff and Perry (18) was used. Slides were first stained in Hoechst fluorescence dye H 33258 (1 μg/ml of 2× SSC) for 15 min, rinsed with distilled water, air dried, then exposed to long-wave UV (360 nm) for 1 hr (the 2× SSC-covered slides were positioned 20 cm from the UV source). After being rinsed and air dried, the slides were stained in 7% Giemsa in Sørensen phosphate buffer (0.07 M KH_2PO_4/0.07 M Na_2HPO_4), pH 6.8, rinsed with distilled water, and air dried. The slides were analyzed with a Zeiss photomicroscope III using bright field and a Planapo objective 63×/1.4. For better visualization of the banding pattern, a green interference filter (546 nm) was used. The silver grains were more easily identified without this filter.

RESULTS

Cell synchronization with BrdUrd consistently yielded a high percentage of prophase and prometaphase mitoses. About 60–70% of all mitoses were in this stage, and, after banding, up to 1,000 bands per haploid set of chromosomes were clearly visible. About 90% of the mitoses were G-banded; the rest mostly gave a R-banding pattern. The procedure used gave a direct visualization of silver grains from the labeled probe together with the distinct G-banding chromosome pattern (Fig. 1). The banding pattern of the metaphase spreads was best observed when a green interference filter was used (Fig. 1, karyotype; Fig. 2 *Left*). However, the silver grains were best visualized without this filter (Fig. 1 *Inset*; Fig. 2 *Right*). Seventy-five metaphases (Fig. 3) containing complete, optimally banded chromosome spreads with precise grain locations were scored for each probe. Grains were scored only if they were located on the chromosome or were touching at least one chromatid. Using these criteria for each probe, we found a specific and consistent chromosome site labeled in about 20–30% of the metaphases, usually on one chromosome but seldom on both homologs. These specific grains were more often found in prometaphase than in metaphase chromosomes. Their distribution ranged over 2–3 chromosome bands with a clear peak for one band, which we determined as the site of the gene locus (Fig. 3). Generally the labeling resulted in one silver grain on the chromosome or sometimes one grain on each chromatid (after 7–10 days of exposure). The level of nonspecific background labeling was relatively low (about 3 grains per metaphase) with unspecific localizations (Fig. 3).

We localized *AMY* at 1p21 (close to 1p13) (Fig. 3). In 37% of the metaphases, grains were at this band which represented 70% of all grains on chromosome 1 and 16% of the total grains counted. The *AMY*-containing probe was the only probe that occasionally showed two or more grains on one or both chromatids (Fig. 4). *POMC* was mapped at 2p23 (close to 2p24) (Fig. 3). Twenty-six percent of the metaphases showed specific labeling in this area, which represents >80% of all grains on chromosome 2. Twelve percent of all grains counted were localized in this area on chromosome 2. We localized the *SST* at 3q28 (close to 3qter). Twenty percent of all metaphases were positive for this region, and about 70% of all grains on chromosome 3 were found at this site. This represented 10% of the total grains. The chromosomal position of *D3S1* that recognizes a restriction site polymorphism was localized to the band 3q12 (at the border to 3q11) (Fig. 3). Thirty-three percent of the metaphases had grains at these bands on chromosome 3, and

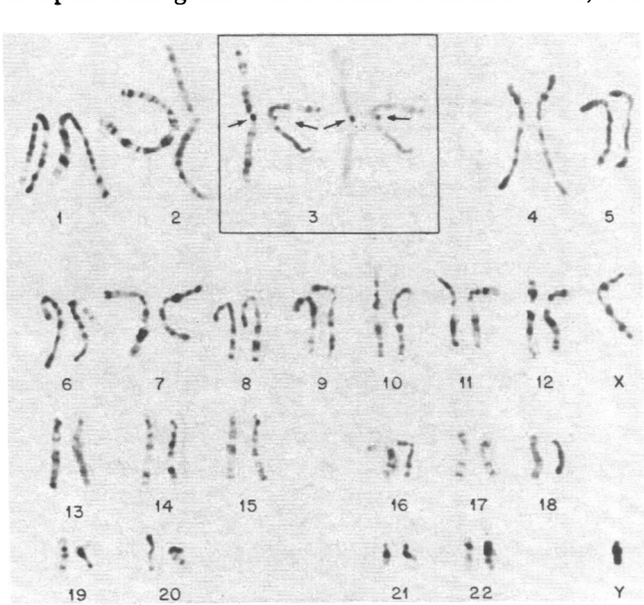

FIG. 1. *In situ* hybridization with the *D3S1* probe, G-banded karyotype of prometaphase chromosomes, and labeling of both homologs in the 3q12 region. (*Inset*) Both chromosomes 3 focused on the banding pattern with a green interference filter (*Left*) or on the silver grains (*Right*).

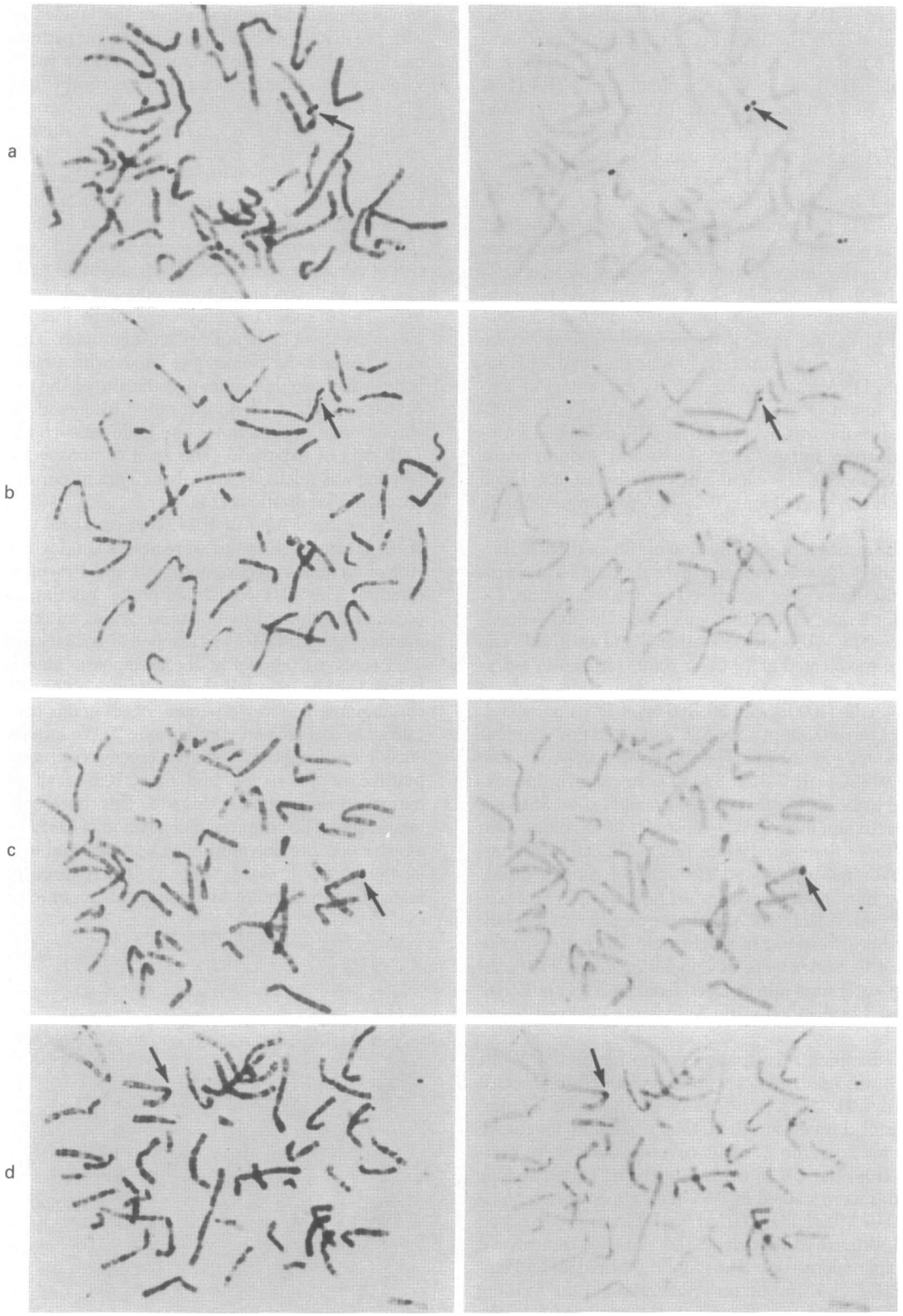

FIG. 2. *In situ* hybridization with the four different probes: *AMY* (*a*), *POMC* (*b*), *SST* (*c*), and *D3S1* (*d*). G-banded metaphases: with a green interference filter (546 nm), the banding pattern is more visible (*Left*); without the filter, the silver grains are more easily identified (*Right*).

over 80% of the grains on chromosome 3 were in this region. Grains at this site represent 15% of the total grains.

DISCUSSION

For a precise chromosomal localization of a gene by *in situ* hybridization, the methodology should be optimized to have chromosomes divided into the largest number of defined bands possible with a minimum of silver grain scatter. BrdUrd is a cell-synchronizing agent that allows the isolation of large prometaphase chromosomes with a distinct and detailed banding pattern. BrdUrd is incorporated only in early replicating R-bands because the BrdUrd block occurs in the middle of S phase, and

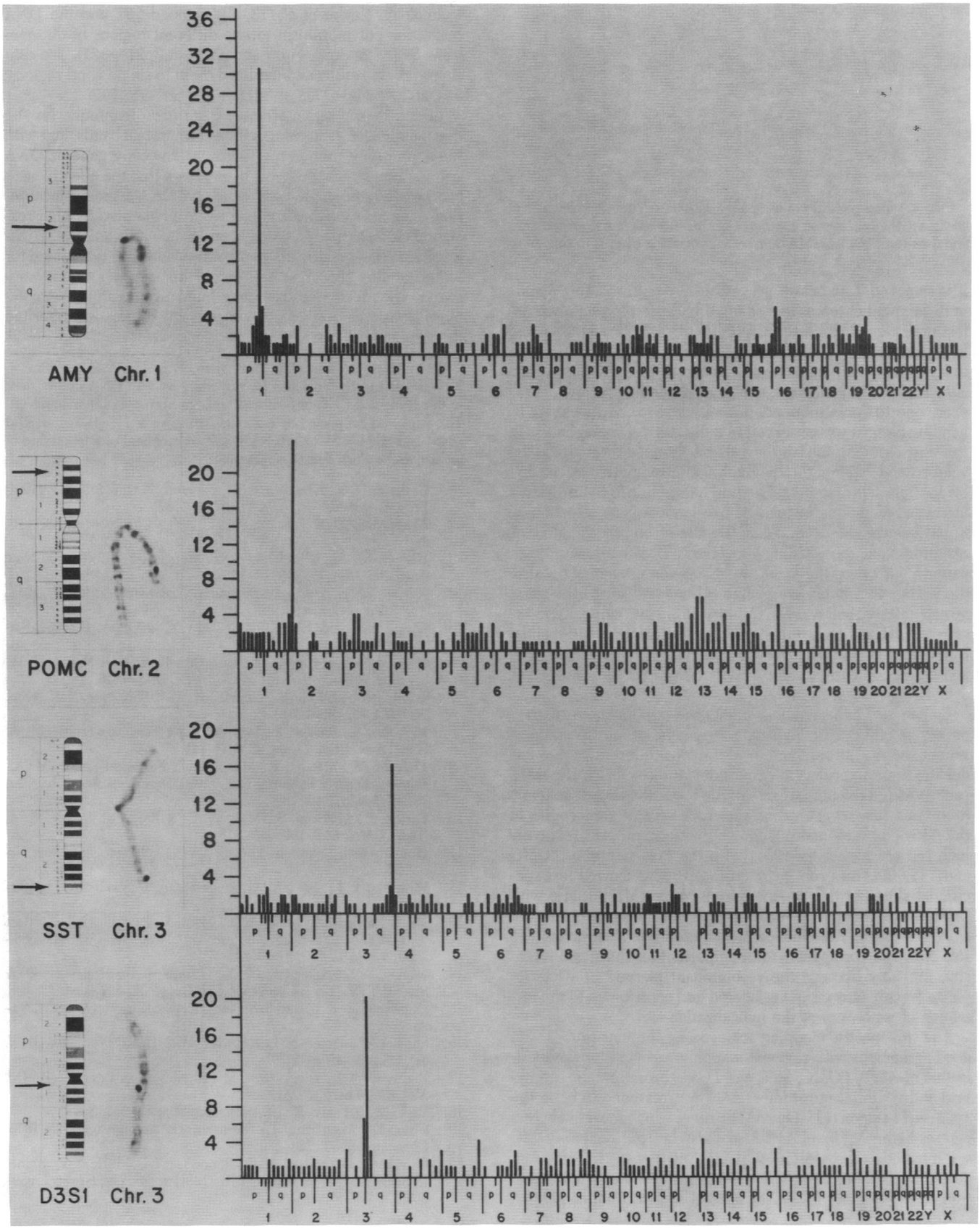

FIG. 3. (*Right*) Diagram showing the grain distribution in 75 metaphases for each of the four different probes (*AMY*, *POMC*, *SST*, and *D3S1*). The *x* axis represents the chromosomes in their relative size proportion; the *y* axis gives the number of silver grains. For each probe a distinct chromosome region has a highly significant amount of labeling in comparison to the unspecific background grains found on all chromosomes. (*Left*) The specific site of hybridization for each probe (*AMY* at 1p21, *POMC* at 2p23, *SST* at 3q28, and *D3S1* at 3q12) is shown together with a representative labeled and banded chromosome.

FIG. 4. Two partial G-banded metaphases. The *AMY* probe hybridizes to the 1p21 region (arrows). Occasionally one or two grains were found on one chromatid (*Left*) or both chromatids (*Right*) in this area.

it is removed 7 hr before harvesting the culture. This results in differential chromosome staining because the photolytic effect of UV on BrdUrd-substituted chromatin, when enhanced by a photosensitive dye (Hoechst 33258), causes a reduced affinity in substituted areas for components of the Giemsa stain (19). This technique results in a good G-banding pattern. However, due to an infrequent, incomplete BrdUrd block, an R-banding pattern was observed in a minority of metaphases. In this way, chromosome banding can be obtained through the developed and fixed autoradiographic emulsion after *in situ* hybridization. Chromosomal banding before *in situ* hybridization has been recommended to avoid bias in metaphase selection (8, 9) but has the disadvantage of the loss of chromosomal DNA resulting from the banding procedure. BrdUrd does not apparently influence the *in situ* hybridization efficiency because the probes that hybridize to light G-bands (where BrdUrd is incorporated), such as *POMC* and *D3S1*, gave comparable results to *AMY* and *SST* probes, which hybridize to dark G-bands. In addition, preliminary studies with the *D3S1* probe showed no decrease in chromosome site-specific silver grains whether or not BrdUrd was used.

Our *in situ* hybridization efficiency was similar to that reported by others utilizing single-copy gene probes. A comparison of the results obtained from the four different probes under the same hybridization conditions indicates that the *AMY* probe yielded the highest signal. *AMY* was the largest genomic probe in a phage vector, and there are several gene copies in the *AMY* chromosomal region. The background was relatively high but not higher than that with the *POMC* probe. *POMC* and *SST*, the two probes cloned in pBR322, showed less specific labeling not only in comparison with *AMY* but also with *D3S1*, a single-copy DNA probe of about the same insert size as *POMC* and *SST* but cloned in bacteriophage λ as vector. The size of the vector appears to be important for the signal intensity, probably because the recombinant plasmid or bacteriophage (vector plus insert) is labeled and contributes to the formation of a network at the hybridization site.

The site-specific mapping achieved for the four probes is in good agreement with regional mapping reported for the localization of *AMY*, *POMC*, *SST*, and *D3S1*. From somatic cell hybrid and gene-linkage studies, *AMY* was reasoned to be in the 1p22→q11 region (11). Our *AMY* probe, which most likely recognizes both *AMY1* and *AMY2*, maps to the 1p21 region. *POMC* was mapped to chromosome 2 by Owerbach *et al.* (20), who suggested an assignment on the short arm. Our *in situ* hybridization study localizes *POMC* at 2p23. Using recombinant DNA technology, Naylor *et al.* (13, 14) mapped *SST* and the DNA restriction polymorphism to two different regions of chromosome 3 (*SST* at 3q21→qter and *D3S1* at 3p21→q21). We confirmed these results and localized them to single band regions: *SST* at 3q28 and *D3S1* at 3q12.

In situ hybridization strategy is an ideal technique for the fine mapping of genes to specific chromosomal bands, and with the appropriate probe, any single- or low-copy gene or DNA segment can be mapped. We have shown that the effectiveness and precision of single-copy gene and DNA segment localization using *in situ* hybridization can be augmented by high-resolution chromosome preparation and banding techniques. High-resolution site-specific mapping of human genes is essential for understanding the organization and linear order on chromosomes. The precise localization of human genes and DNA polymorphisms will be important in linkage studies for inherited diseases and cancer.

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1. Gerhard, D. S., Kawasaki, E. S., Bancroft, F. C. & Szabo, P. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 3755–3759.
2. Harper, M. E. & Saunders, G. F. (1981) *Chromosoma* **83**, 431–439.
3. Harper, M. E., Ullrich, A. & Saunders, G. F. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 4458–4460.
4. Harper, M. E., Barrera-Saldaña, H. A. & Saunders, G. F. (1982) *Am. J. Hum. Genet.* **34**, 227–234.
5. Kirsch, I. R., Morton, C., Nakahara, K. & Leder, P. (1982) *Science* **216**, 301–303.
6. Neel, B. G., Suresh, C. Z., Chaganti, R. S. K. & Hayward, W. S. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 7842–7846.
7. Trent, J. M., Olson, S. & Lawn, R. M. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 7809–7813.
8. Malcolm, S., Barton, P., Murphy, C. & Ferguson-Smith, M. A. (1981) *Ann. Hum. Genet.* **45**, 135–141.
9. Malcolm, S., Marton, P., Murphy, C., Ferguson-Smith, M. A., Bentley, D. L. & Rabbitts, T. H. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 4957–4961.
10. Barton, P., Malcolm, S., Murphy, C. & Ferguson-Smith, M. A. (1982) *J. Mol. Biol.* **156**, 269–278.
11. Human Gene Mapping Workshop VI (1982) *Cytogenet. Cell Genet.* **32**.
12. Whitfeld, P. L., Seeburg, P. H. & Shine, J. (1982) *DNA* **1**, 133–143.
13. Naylor, S. L., Sakaguchi, A. Y., Shen, L.-P., Bell, G. I., Rutter, W. J. & Shows, T. B. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 2686–2689.
14. Naylor, S. L., Sakaguchi, A. Y., Gusella, J. F., Housman, D. & Shows, T. B. (1982) *Cytogenet. Cell Genet.* **32**, 302 (abstr.).
15. Dutrillaux, B. & Viegas-Pequignot, E. (1981) *Hum. Genet.* **57**, 93–95.
16. Shen, L.-P., Pictet, R. L. & Rutter, W. J. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 4575–4579.
17. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol.* **113**, 237–251.
18. Wolff, S. & Perry, P. (1974) *Chromosoma* **48**, 341–353.
19. Goto, K., Akematsu, T., Shimazu, H. & Sugiyama, T. (1975) *Chromosoma* **53**, 223–230.
20. Owerbach, D., Rutter, W. J., Roberts, J. L., Whitfeld, P., Shine, J., Seeburg, P. H. & Shows, T. B. (1981) *Somat. Cell Genet.* **7**, 359–369.