SHORT COMMUNICATION

Fluorescence *in Situ* Hybridization Establishes the Order cen–DXS28(C7)–DXS67(B24)–DXS68(L1)–tel in Human Chromosome Xp21.3

BARBARA J. TRASK,*,[1] HILLARY F. MASSA,* AND MARGIT BURMEISTER†

*Human Genome Center, Biomedical Sciences Division, Lawrence Livermore National Laboratory, Livermore, California 94550; and †Mental Health Research Institute, University of Michigan School of Medicine, Ann Arbor, Michigan

Received October 28, 1991; revised January 2, 1992

We report here on the order of three DNA markers, C7, B24, and L1, based on the arrangement of their fluorescently labeled hybridization sites in interphase cell nuclei. The three markers map distal to the Duchenne muscular dystrophy (DMD), glycerol kinase deficiency (GKD), and adrenal hypoplasia (AHC) loci on human chromosome Xp21.3. Their order has been a matter of controversy. In interphase chromatin, B24 maps between C7 and L1. We estimate from interphase distance that C7 and L1 are 300–500 kb apart. When the three markers are hybridized to interphase cells of Nijmegen1, a patient with DMD, GKD, and AHC, only C7 appears to be deleted, rather than both C7 and L1, as had been reported elsewhere. C7 is also the only one of the three markers deleted in several other DMD patients studied by others. The deletion results indicate that C7 is the most proximal of the three markers and allow the trio of ordered probes to be oriented on the chromosome: cen–C7(DXS28)–B24(DXS67)–L1(DXS68)–tel.

The three DNA markers, C7 (DXS28), B24 (DXS67), and L1 (DXS68), map distal to the AHC, GKD, and DMD loci in human chromosome Xp21 (4). These probes are frequently used to define the extent of deletions in patients with contiguous gene syndromes involving these loci. They also aid in long-range mapping of this clinically important chromosomal region.

The order of these three markers has been a matter of controversy. A number of publications have addressed this issue, with conflicting results and conclusions (1–3, 8–10, 14). In fact, of the six possible permutations of the order of these three loci, only one has not yet been put forth in the literature.

Pulsed-field gel electrophoresis (PFGE) analysis shows that an ≈1.1-Mb partial *Bss*HII restriction fragment is shared by L1 and B24, but not C7, and that B24 and C7, but not L1, hybridize to a common 800-kb *Bss*HII fragment (3). These PFGE results suggest the order C7–B24–L1, but provide no orientation of the cluster relative to the aforementioned disease loci. Segregation analysis in a DMD family suggests that B24 maps between C7 and DMD (8). This conclusion is in conflict with the analyses of patients with deletions involving AHC, GKD, and DMD. The deletions in three such patients include C7, but not L1 or B24 (1, 9, 10). These deletion results indicate that C7 is the most proximal (closest to DMD) of the three markers. However, the deletion of one patient, referred to as Nijmegen1, has been reported to include both C7 and L1, but not B24 (14). The result on this patient is inconsistent with the PFGE results.

To resolve these discrepancies, we employed a different approach, interphase chromatin mapping, to derive the order of C7, B24, and L1. In this approach, the order of probes is derived from the relative positions of their fluorescently labeled hybridization sites in interphase nuclear chromatin. The distance between hybridization sites in interphase has been shown to increase with the genomic distance separating DNA probes in the range from 50 kb to at least 1 Mb (7, 12, 13). Using interphase mapping, we show here that B24 maps between C7 and L1. In addition, we have used fluorescence *in situ* hybridization (FISH) to reexamine the deletion in the Nijmegen1 patient. We find that the deletion does not include L1 as previously reported and is thus consistent with the map cen–DMD–C7–B24–L1–tel.

The following probes were labeled with either biotin-dUTP or digoxigenin-dUTP using a nick-translation kit (BRL). L1 is a cosmid clone isolated by hybridization to probe L1-4 (3). C7 is a 15 to 20-kb λ clone kindly provided by H. Zoghbi (Baylor College of Medicine) and isolated by hybridization of C7 (from ATCC) to a human genomic library cloned in λ-DASH (15). B24 is pooled DNA from two phage clones, HXB24A and HXB24B, which contain overlapping inserts of ≈15 kb and were obtained by screening a EMBL3 human genomic library (constructed and kindly provided by G. Benzi and A.-M. Frischauf, ICRF, London) (unpublished results; (5)) with the probe B24. Probes L1-4 and B24 (6) were kindly provided by L. Kunkel (Childrens Hospital, Boston).

The probes were hybridized together in various combinations to G1 interphase nuclei from normal male fibro-

[1] To whom correspondence should be addressed at L-452, Biomedical Sciences Division, Lawrence Livermore National Laboratory, P. O. Box 5507, Livermore, CA 94550.

GENOMICS 13, 455–457 (1992)
0888-7543/92 $5.00

blast culture cells (HSF7), as described elsewhere (13). In some experiments, the slides were incubated in 0.08 μg/ml proteinase K for 7 min at 37°C before denaturation to decrease nonspecific signals (11). After hybridization and washing, biotin- and digoxigenin-labeled probes were detected with avidin-Texas Red and FITC-conjugated antibodies, respectively, as described (13), with minor modifications (Trask *et al.*, manuscript in preparation). Slides were mounted in an antifade solution containing the DNA stain DAPI. Texas Red and FITC fluorescence were viewed simultaneously through a dual band pass filter (Omega, Brattleboro, VT) on a Zeiss Axioskop microscope (100×, 1.3 NA).

The order of C7, B24, and L1 was first determined by hybridizing the three probes simultaneously to male G1 interphase nuclei. The site of one probe was labeled in a color distinct from the other two probe sites (Fig. 1a). The identity of the odd-colored site was varied among experiments. The position of the odd-colored probe was scored as either inside or outside the area between the two like-colored probes in ≈100 nuclei (Table 1). In all experiments, the most frequently observed arrangement of hybridization sites was consistent with the order C7–B24–L1.

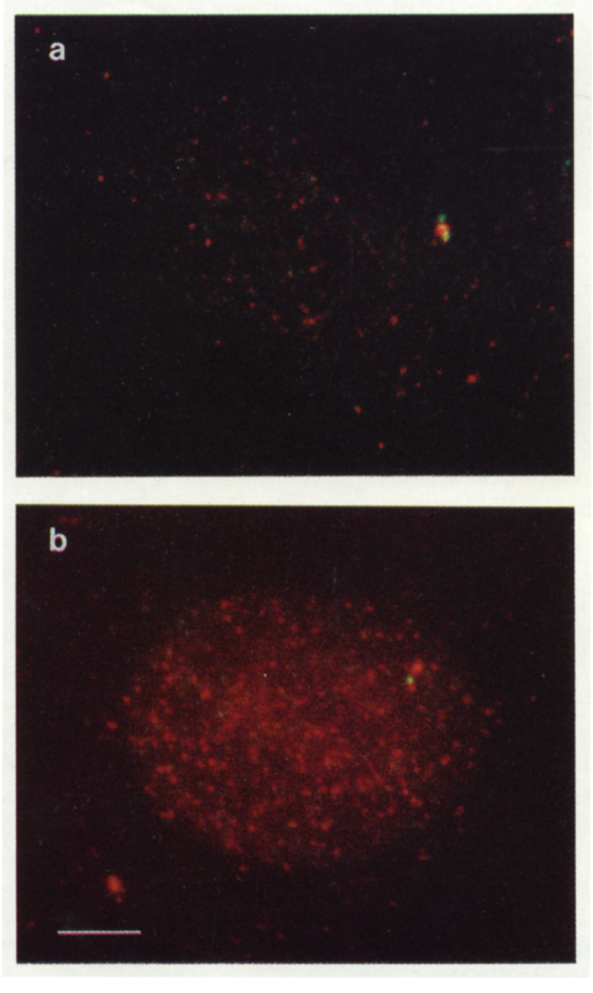

FIG. 1. FISH analysis of interphase nuclei from normal male and Nijmegen1 patient. (**a**) Hybridization of C7 and L1 (both yellow-green) and B24 (orange-red) to G1 interphase nucleus of a normal male. B24 falls between C7 and L1 in this and the majority of other nuclei (see Table 1). (**b**) Hybridization of L1 (yellow-green) and B24 (orange-red) to a nucleus from the Nijmegen1 cell culture. L1 is not deleted in this patient as evidenced by the presence of a green hybridization site in this and >93% of the other nuclei (see Table 2). Bar = 5 μm.

TABLE 1

Probe Order in Interphase Nuclei[a]

Color label[b] of probes			Observed order[c] (number of nuclei)	
C7	B24	L1		
			RRG (G outside)	RGR (G inside)
G	R	R	**66**	33
R	G	R	39	**65**
R	R	G	**65**	36
			GGR (R outside)	GRG (R inside)
R	G	G	**57**	31
G	R	G	42	**79**
G	R	G[d]	64	**140**
G	G	R	**74**	38

[a] Three probes hybridized simultaneously to G1 interphase nuclei from fibroblast cell culture of normal male. The slides bearing each probe combination were coded before scoring at the microscope.

[b] G, green; R, red.

[c] The position of the odd-colored probe was scored as either "inside" or "outside" a circular area between the other two hybridization sites. The diameter of this circular area was defined as the line connecting the two like-colored sites. The values supporting the order C7–B24–L1 are highlighted in bold type in each row.

[d] Results of a duplicate experiment on a different nuclear preparation of the same individual.

Probe order was also derived from the relative distance measured between probes in interphase chromatin. Here, the probes were hybridized in pairs to G1 nuclei. One probe site in each pair was labeled in green and one in red. Photographic slides were taken of 80–140 randomly selected nuclei per probe pair from coded slides. The distances between red and green hybridization sites were measured from projected (1 μm enlarged to 1.4 cm) images of coded photographic slides (12). The mean interphase distance between L1 and C7 (0.67 ± 0.03 μm; ±SEM) was significantly greater than the distance between either C7 and B24 (0.53 ± 0.02 μm) or B24 and L1 (0.51 ± 0.03 μm). The relative distances again indicate that B24 lies between C7 and L1.

The FISH results are inconsistent with the published results on Nijmegen1, a patient with a deletion causing DMD, GKD and AHC and described to include C7 and L1, but not B24. We used FISH to reexamine the deletion in interphase cells from this patient. The cells were kindly provided by G.-J. van Ommen and M. Wapenaar (University of Leiden) and H. H. Ropers and F. Oerlemans (University of Nijmegen). B24 (red) was paired with either C7 or L1 (green) (Fig. 1b), and the number of red and green hybridization sites was scored in >100 nuclei (Table 2). The results confirm that C7 is deleted in this patient but show that both L1 and B24 are retained.

Interphase distance and probe ordering place B24 between C7 and L1. This order assumes a direct correla-

TABLE 2

DNA Probes Hybridizing to Nijmegen1 Patient Nuclei[a]

Probe pair		Green (G) and red (R) sites/nucleus (number of nuclei)			
Green	Red	1 G/1 R	0 G/1 R	1 G/0 R	0 G/0 R
L1	B24	96	6	4	1
C7	B24	0	100	0	0

[a] Two probes hybridized simultaneously; sites labeled in indicated colors.

tion between interphase and genomic distance in this chromosomal region. Such a correlation has been observed in a number of chromosomal regions including two on chromosome X ((7, 12, 13), B. Trask *et al.*, manuscript in preparation). The observed order is consistent with previously published PFGE results, which also placed B24 between C7 and L1 (3).

Analysis of deletions in patients with DMD, GKD, and AHC provides orientation of the C7–B24–L1 trio with respect to these more proximal disease loci. C7 is the most proximal of the three markers. C7 is deleted, but L1 and B24 are retained in three described patients (1, 9, 10). Our reexamination by FISH of the Nijmegen1 patient shows that his deletion also includes C7, but does not include B24 or L1, as had been reported previously. Difficulties with early stocks of the L1 phage (6) may have led to the original erroneous results (14). Nijmegen1 is apparently not deleted for L1-4 (G.-J. van Ommen, personal communication), the subclone used to identify the L1 cosmid for our FISH studies.

We estimate that L1 and C7 are 300–500 kb apart by comparing the mean interphase distance between them (0.67 μm) to interphase distances measured between probes in other well-characterized chromosomal regions ((7, 13), B. Trask *et al.*, manuscript in preparation). This estimate assumes that, after hybridization, DNA is similarly condensed in different regions of the genome over this distance range. The distance estimate is consistent with the 300- to 1500-kb separation estimated from PFGE results (3).

In the course of the last 5 years, five of the six possible orders of C7, B24, and L1 relative to DMD have been put forth in the literature. We now conclude that the sixth and as yet unchampioned order, cen–DMD–C7–B24–L1–tel, is supported by FISH interphase analyses, PFGE results, and deletion analyses.

ACKNOWLEDGMENTS

We thank G.-J. van Ommen, M. Wapenaar, H. H. Ropers, and F. Oerlemans for the Nijmegen1 cells, E. R. B. McCabe for helpful suggestions, and H. Zoghbi, G. Benzi, A.-M. Frischauf, and L. Kunkel for probes. Work at LLNL was performed under the auspices of U.S. D.O.E. under Contract W-7405-ENG-48 with support from National Institutes of Health Grant RO1 HG-00256-01.

REFERENCES

1. Bartley, J., and Gies, C. (1989). A Xp21 deletion assigns locus DXS28 (C7) proximal to DXS68 (L1.4) and DXS67 (B24) and has the proximal breakpoint in the intron 3′ to the first exon of DMD-8 (DMD exon 47). *Cytogenet. Cell Genet.* **51:** 958.
2. Burmeister, M., and Lehrach, H. (1986). Long-range restriction map around the Duchenne muscular dystrophy gene. *Nature* **324:** 582–585.
3. Burmeister, M., Monaco, A. P., Gillard, E. F., van Ommen, G. J. B., Affara, N. A., Ferguson-Smith, M. A., Kunkel, L. M., and Lehrach, H. (1988). A 10-megabase physical map of human Xp21, including the Duchenne muscular dystrophy gene. *Genomics* **2:** 189–202.
4. De Martinville, B., Kunkel, L. M., Bruns, G., Morle, F., Koenig, M., Mandel, J. L., Horwich, A., Latt, S., Gusella, J. F., Housman, D., and Francke, U. (1985). Localization of DNA sequences in region Xp21 of the human X chromosome: Search for molecular markers close to the Duchenne muscular dystrophy locus. *Am. J. Hum. Genet.* **37:** 235–249.
5. Frischauf, A. M., Lehrach, H., Poustka, A., and Murray, N. (1983). Lambda replacement vectors carrying polylinker sequences. *J. Mol. Biol.* **170:** 827–842.
6. Kunkel, L. M., Lalande, M., Monaco, A. P., Flint, A., Middlesworth, W., and Latt, S. A. (1985). Construction of a human X-chromosome-enriched phage library which facilitates analysis of specific clones. *Gene* **33:** 251–258.
7. Lawrence, J. B., Singer, R. H., and McNeil, J. A. (1990). Visual resolution of different distances with the human dystrophin gene in nuclei and chromosomes. *Science* **249:** 928–932.
8. Norby, S., and Schwartz, M. (1987). Assignment of the locus order DXS28–DXS67–DMD as a spin-off from diagnostic DNA marker analysis in a family with Duchenne muscular dystrophy. *Clin. Genet.* **31:** 192–197.
9. Stuhrmann, M., Heilbronner, H., Reis, A., Wegner, R. D., Fischer, P., and Schmidtke, P. (1991). Characterization of a Xp21 microdeletion syndrome in a 2-year-old boy with muscular dystrophy, glycerol kinase deficiency and adrenal hypoplasia congenita. *Hum. Genet.* **86:** 414–415.
10. Towbin, J. A., Chamberlain, J. S., Wu, D., Pillers, D. M., Seltzer, W., and McCabe, E. R. B. (1990). DXS28 (C7) maps centromeric to DXS68 (L1-4) and DXS67 (B24) by deletion analysis. *Genomics* **7:** 442–444.
11. Trask, B., and Pinkel, D. (1989). Fluorescence in situ hybridization with DNA probes. *In* "Methods in Cell Biology" (Z. Darzynkiewicz and H. Crissman, Eds.), Vol. 33, pp. 383–400, Academic Press, San Diego.
12. Trask, B. J., Pinkel, D., and van den Engh, G. J. (1989). The proximity of DNA sequences in interphase cell nuclei is correlated to genomic distance and permits ordering of cosmids spanning 250 kilobase pairs. *Genomics* **5:** 710–717.
13. Trask, B. J., Massa, H. F., Kenwrick, S., and Gitschier, J. (1991). Mapping of human chromosome Xq28 by 2-color fluorescence in situ hybridization of DNA sequences to interphase cell nuclei. *Am. J. Hum. Genet.* **48:** 1–15.
14. Van Ommen, G. J. B., Verkerk, J. M. H., Hofker, M. H., Monaco, A. P., Kunkel, L. M., Ray, P., Worton, R., Wieringa, B., Bakker, E., and Pearson, P. L. (1986). A physical map of 4 million bp around the Duchenne muscular dystrophy gene on the human X-chromosome. *Cell* **47:** 499–504.
15. Zoghbi, H. Y., Ballantyne, C. M., O'Brien, W. E., McCall, A. E., Kwiatkowski, T. J., Ledbetter, S. A., and Beaudet, A. L. (1990). Deletion and linkage mapping of eight markers from the proximal short arm of chromosome 6. *Genomics* **6:** 352–357.