Jpn J Human Genet 42, 525-532, 1997

FISH MAPPING OF A TRANSLOCATION BREAKPOINT AT 6q21 (OR q22) IN A PATIENT WITH HETEROTAXIA

Rumiko KATO,[1] Naomichi MATSUMOTO,[1] Masahiro FUJIMOTO,[1,2] Motoi NAKANO,[1,3] Yusuke NAKAMURA,[4] and Norio NIIKAWA[1,*]

[1] *Department of Human Genetics,* [2] *Second Department of Surgery, and* [3] *Department of Plastic and Reconstructive Surgery, Nagasaki University School of Medicine, Sakamoto 1-12-4, Nagasaki 852, Japan*
[4] *Human Genome Center, Institute of Medical Sciences, The University of Tokyo, Tokyo 108, Japan*

Summary Heterotaxia is a congenital lateralization defect of visceral organs. As several single-genes that act on the formation of left-right asymmetry during embryogenesis have been identified in animals, a defect in the similar system may play a role in heterotaxia in man. We previously reported a Japanese girl with heterotaxia associated with a *de novo* balanced translocation (6;18)(q21 or q22;q21.3 or q22). In the present study, based on a hypothesis that one of the putative situs-determining genes is disrupted at a breakpoint of the translocation, we first isolated a yeast artificial chromosome (YAC) clone covering a breakpoint, 6q21 (or q22) of the translocation. Then, using STSs mapped on the YAC, we isolated bacterial artificial chromosome (BAC) clones spanning the breakpoint. FISH analysis using the BAC clones as probes revealed that the breakpoint is confined to a segment between two STS loci, WI-4066 and the CHLC.GATA6B06.192, within a genetic distance of 1.4 cM. The human connexin43 gene was not disrupted in our patient, although mutations of this gene have been reported in patients with complex heart disease and heterotaxia. The molecular localization of the translocation breakpoint in our patient may contribute to the positional cloning of a putative heterotaxia gene.

Key Words FISH, mapping, STS, t(6q;18q), heterotaxia

INTRODUCTION

Heterotaxia is a lateralization defect with an incidence of about one in 10,000 persons (Torgersen, 1949), and characterized by an inverse position of thoracic and/or abdominal organs, occurring in early embryonic development. It is often associated with complex heart diseases. Different inheritance patterns of heterotax-

Received July 23, 1997; Accepted August 11, 1997.
* To whom correspondence should be addressed.

ia, including autosomal recessive, autosomal dominant, and X-linked recessive modes, suggest that the normal formation of left-right asymmetry is determined by multiple genes. Although genes or loci, such as *cAct-RIIa, Shh, cNR-1, nodal, lefty, inv* and *iv*, have been identified to play important roles in the determination of the left-right asymmetry in the chicken and/or murine (Levin *et al.*, 1995; Meno *et al.*, 1996; Collignon *et al.*, 1996; Lowe *et al.*, 1996; Brueckner *et al.*, 1989; Yokoyama *et al.*, 1993), it remains to be seen whether defects of their human homologs are causative for heterotaxia. The human connexin43 gene (*GJA1*) has recently been noted as a cause for one type of heterotaxia. *GJA1* mutations were identified in 6 of 30 patients with complex heart diseases and heterotaxia (Britz-Cunningham *et al.*, 1995). However, as similar studies by two other groups failed to identify any mutations in a total of 40 patients (Casey and Ballabio, 1995; Splitt *et al.*, 1995), contribution of *GJA1* to the diseases remains still unconfirmed.

We previously reported a sporadic case of heterotaxia associated with *de novo* balanced translocation: 46,XX,t(6;18)(q21 or q22;q21.3 or q22) (Kato *et al.*, 1996). We hypothesized that a putative situs-determining gene is disrupted in the patient by the translocation. This paper deals with a molecular mapping of a breakpoint (6q21 or q22) using a yeast artificial chromosome (YAC) clone and its corresponding bacterial artificial chromosome (BAC) clones. As *GJA1* was assigned at around the breakpoint (Kato *et al.*, 1997), we also investigated on whether the gene is disrupted in this patient.

MATERIALS AND METHODS

Isolation of YAC clones. According to a YAC contig map by Centre d'Etude du Polymorphisme Humain (CEPH) (Chumakov *et al.*, 1995), 33 CEPH-YAC clones were chosen from a region covering a breakpoint, 6q21 or q22, of a translocation in the patient. Alu-PCR was performed as described previously (Kuwano *et al.*, 1992). DNA from each YAC was amplified for 30 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 4 min, using the primers (5′-3′): 2484, AGGAGTGAGCCACCGCACCCAGCCC and PDJ34, TGAGC(C/T)(G/A)(A/T)GAT(C/T)(G/A)(C/T)(G/A)CCA(C/T)TGCACTCCAGCCTGGG. The PCR products were combined and used for fluorescence *in situ* hybridization (FISH) analysis.

Isolation of BAC clones. A human BAC library (Research Genetics, USA) was screened by means of PCR, according to the manufacturer's protocol. Five sequence-tagged-site (STS) primers that are corresponding to or near the YAC 798G12 (Hudson *et al.*, 1995) were used. Sequences (5′-3′) of forward/reverse primers are as follows: CHLC.ATA16B01, CAGTTTGTTCAGAAATTAAAAACG/CAATCTCACTATAAACCTGACCG; WI-1240, CCATAAAATCCAACCATGGA/TCATGAAGGGCACACATATG; WI-4066, TCTGTGCAAACCACCATCAT/GAGAGCATTGGGTTCTGTAAGC; CHLC.GATA6B06.192, TC-

CAGGTTGCTGAAAAAGAC/CAATTGCAAGGATACGGAAC; WI-4792, CTCAAACATGATAGCCAAAAGC/CCACCCATCCATCTACCAAA. PCR was 30 cycled at 94°C for 30 sec, at 55-63°C for 30 sec and at 72°C for 30 sec using GeneAmp System 9600 (Perkin-Elmer, USA).

Fluorescence in situ hybridization (FISH). Chromosome preparations were made from peripheral blood and EB-transformed lymphoblasts. Isolated YAC or BAC clones were labeled with biotin-16-dUTP (Boehringer-Mannheim, Germany) by nick-translation. FISH was performed using human Cot I DNA as a competitor, and FISH signals were detected with FITC-avidin (Boehringer-Mannheim) as described previously (Ohta *et al.*, 1993).

Southern blot hybridization. In order to try to detect rearranged *GJA1*-fragments that would be associated with the translocation in our patient, Southern blot analysis was performed. Genomic DNAs from the patient and from six karyotypically normal individuals were digested with *Eco*RI or *Hin*dIII, separated on an agarose gel, and transferred to nylon membranes. A GJA1 probe designed to cover 86% of the entire sequence of *GJA1*-cDNA (Fishman *et al.*, 1991) was generated by PCR using the following primer pair (5′-3′): GJA1f, ACTGGAGC-GCCTTAGGCAAA; GJA1r, CTAGATCTCCAGGTCATCAG. The probe was hybridized to the DNA at 65°C.

RESULTS

FISH analysis on normal metaphase chromosomes revealed that 22 of 33 YAC clones gave signals on the long-arm of homologous chromosomes 6, while the remaining 11 had no signals. Eleven of 22 clones were chimeric or have Alu sequences, and showed additional signals on other chromosomes. When using the patient's chromosomes, 9 clones gave signals on both the normal chromosome 6 and the der(6) chromosome, and 12 clones on both the normal and der(18) chromosomes, indicating that they are mapped proximal and distal to the breakpoint, respectively. Only one clone, 798G12, gave FISH signals on both der(6) and der(18) chromosomes, as well as the normal chromosome 6 (Fig. 1). The result suggested that this YAC spans the breakpoint in our patient. However, as 798G12 is a chimeric clone and contains a genomic fragment of a pericentromeric region of chromosome 4 (Hudson *et al.*, 1995), we did not use it for further analysis.

According to information on 5 STSs at around the breakpoint (Hudson *et al.*, 1995), BAC clones were isolated from a BAC library. Using two (WI-1240 and WI-4066) of three STSs that correspond to 798G12, two BACs (371A24 and 86A6) were isolated, respectively. Another STS, CHLC.ATA16B01 failed to detect any BAC clones. FISH analysis using 371A24 and 86A6 as probes revealed signals on the normal and der(6) chromosomes of the patient. Three BACs (230J13, 136L6, and 177C16) were isolated by the use of the remaining 2 STSs (CHLC. GATA6B06.192 and WI-4792) which were located distal to WI-4066, respectively

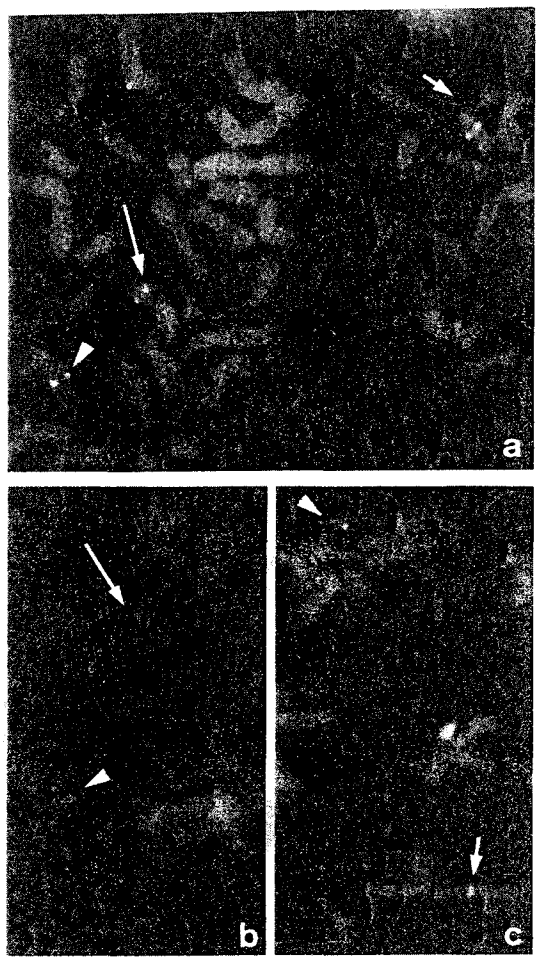

Fig. 1. FISH analysis on the patient's chromosomes. Alu-PCR product from 798G12-YAC gives three signals, each on normal chromosome 6, der(6) and der(18), together with faint signals on the pericentromeric region of both chromosomes 4 due to its chimeric nature (a). Using BAC clones (86A6 and 230J13) as probes, signals appear on the der(6) (b) and on the der(18) (c), respectively, each along with the signals on normal chromosomes 6. Arrowheads, and long- and short-arrows indicate the normal chromosome 6, the der(6), and the der(18), respectively.

(Fig. 2). All 3 these clones gave FISH signals on the normal and der(18) chromosomes of the patient. The findings, along with the STS map (Hudson *et al.*, 1995), indicated that the translocation breakpoint, 6q21 or 6q22, exists between the WI-4066 and CHLC.GATA6B06.192 loci. A genetic distance between the two loci is estimated to be at most 1.4 cM (Dib *et al.*, 1996) (Fig. 2).

Two BAC clones, 342M16 and 326J20, corresponding to *GJA1*, were isolated

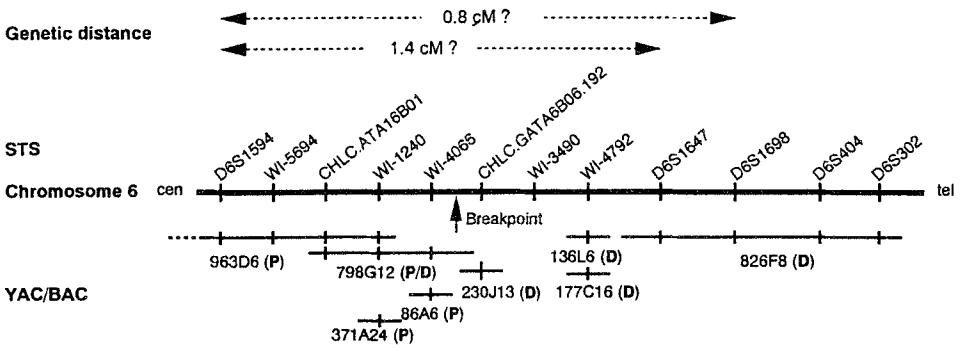

Fig. 2. Schematic presentation of clones located proximal (P) and distal (D) to the breakpoint of the patient.

(Kato *et al.*, 1997). FISH analysis using these clones showed signals on both the normal no. 6 and der(18) chromosomes of the patient. Southern hybridization using the *GJA1*-cDNA as a probe revealed no extra segment in the patient (data not shown). Thus, GJA1 is most likely localized distal to the breakpoint of 6q21 or 6q22.

DISCUSSION

From a total of 33 YACs analyzed, we identified a clone, 798G12, that covers the 6q21 or 6q22 breakpoint. However, this YAC contained an additional human chromosomal segment and was unstable during subcloning, due to its chimeric nature. Instead, we chose BAC-clone isolation as a source to detect a region containing the putative heterotaxia gene. Five BAC clones were isolated from a region corresponding to 798G12, and the breakpoint was successfully assigned between two adjacent STS loci, WI-4066 and CHLC.GATA6B06.192. Genetic distance between the two loci is maximumly 1.4 cM, although the STS order in the region has remained inconclusive (Hudson *et al.*, 1995).

Recent experiments have revealed genes which contribute to the determination of left-right asymmetry of the body. In the chicken, genes such as *activin receptor IIa* (*cAct-RIIa*), *sonic hedgehog* (*Shh*), and *chicken nodal related 1* (*cNR-1*), a member of the TGF-β gene family, are sequentially and asymmetrically expressed around the primitive streak and node during normal embryogenesis (Levin *et al.*, 1995). The murine genes, *nodal* and *lefty* belonging to the TGF-β gene family are also normally expressed in the left half of the node (Meno *et al.*, 1996; Collignon *et al.*, 1996; Lowe *et al.*, 1996). However, in a mutant mouse (*inv*) with "inversion of embryonic turning" who is homozygous for a transgene-induced insertional mutation, both *nodal* and *lefty* are expressed only on the inverse (right) side of the node (Yokoyama *et al.*, 1993). In addition, in another mutant mouse, *inversus viscerum* (*iv*) who has randomized situs with 50% occurrence of heterotaxia, these

genes are also expressed inversely or randomly in the half of the node (Brueckner *et al.*, 1989). Thus, it is likely that the *inv* and *iv* loci, together with *nodal* and *lefty*, play important roles in the determination of the left-right axis in early embryonic development. Since the other locus, *iv*, lies on mouse chromosome 12 and its corresponding human locus is suspected to be at 14q32 (Brueckner *et al.*, 1989), it is less likely that its human homologue contributes to the heterotaxia in our patient. The *inv* is assigned to the mouse chromosome 4, being 5.8-cM apart from the alphapeptide gene (*Tsha*) for glycoprotein hormones and 10-cM from the hexabrachion gene (*Hxb*) (Yokoyama *et al.*, 1993). The human *TSHA* is mapped to human chromosome 6q21.1-q23 (Naylor *et al.*, 1983), and *HXB* to 9q33 (White *et al.*, 1992). Localization of *TSHA* is corresponding to or near the breakpoint region of our patient. Thus, if a segment between *Tsha* and the *inv* locus is conserved in the human genome, the putative human homolog (*INV*) may become a strong candidate gene responsible for the heterotaxia in our patient, although *inv* is a recessive condition and the disease in our patient is presumed to be dominant. Positional cloning of the either mouse and/or human gene will give an answer.

Connexin proteins that constitute the intercellular channels of gap junctions permit transfer of small molecules from cell to cell (Willecke *et al.*, 1990). Connexin43, a member of the connexin family, is a major protein expressed in the heart and may play a role in synchronizing heart contraction. The mouse connexin43 gene is expressed transiently on the ectoderm layer in an early developmental stage (Yancey *et al.*, 1992), indicating that connexin43 may contribute to the embryogenesis by cell to cell communication. In view of these findings, along with the observation of *GJA1* mutations in patients with heterotaxia (Britz-Cunningham *et al.*, 1995) and with the gene localization at around the breakpoint in our girl, a mutation of *GJA1* would have caused her disease. Results of the present FISH and Southern blot analyses almost denied the possibility that the gene is directly disrupted by the translocation in our patient. However, a position effect cannot be ruled out, because causative genes were sometimes localized outside the breakpoints in patients, such as those with campomelic dysplasia (Foster *et al.*, 1994), Greig syndrome (Vortkamp *et al.*, 1991), aniridia (Fantes *et al.*, 1995) and holoprosencephaly (Belloni *et al.*, 1996; Roessler *et al.*, 1996).

Acknowledgments We express our gratitude to Dr. Eiichi Soeda at Riken Gene Bank, Tsukuba Life Science Center, the Institute of Physical and Chemical Research for his technical advice and Dr. Ilya Chumakov at Centre d'Etude du Polymorphisme Humain for providing information of YAC locations. We also thank Dr. Yutaka Yamada for providing the patient's specimen. N.N. was supported in part by Grant-in-Aid for Scientific Research (A) (08307019) and that on Priority Areas (Genome Science) from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

Belloni E, Muenke M, Roessler E, Traverso G, Siegel-Bartelt J, Frumkin A, Mitchell HF, Donis-Keller H, Helms C, Hing AV, Heng HHQ, Koop B, Martindale D, Rommens JM, Tsui L-C, Scherer SW (1996): Identification of *Sonic Hedgehog* as a candidate gene responsible for holoprosencephaly. Nature Genet **14**: 353–356

Britz-Cunningham SH, Shah MM, Zuppan CW, Fletcher WH (1995): Mutations in the connexin43 gap-junction gene in patients with heart malformations and defects of laterality. N Engl J Med **332**: 1323–1329

Brueckner M, D'Eustachio P, Horwich AL (1989): Linkage mapping of a mouse gene, *iv*, that controls left-right asymmetry of the heart and viscera. Proc Natl Acad Sci USA **86**: 5035–5038

Casey B, Ballabio A (1995): *Connexin43* mutations in sporadic and familial defects of laterality. N Engl J Med **333**: 941

Chumakov IM, Rigault P, LeGall I, Bellanné-Chantelot C, Billault A, Guillou S, Soularue P, Guasconi G, Poullier E, Gros I, Belova M, Sambucy J-L, Susini L, Gervy P, Glibert F, Beaufils S, Bui H, Massart C, DeTand M-F, Dukasz F, Lecoulant S, Ougen P, Perrot V, Saumier M, Soravito C, Bahouayila R, Cohen-Akenine A, Barillot E, Bertrand S, Codani J-J, Caterina D, Georges I, Lacroix B, Lucotte G, Sahbatou M, Schmit C, Sangouard M, Tubacher E, Dib C, Fauré S, Fizames C, Gyapay G, Millasseau P, NGuyen S, Muselet D, Vignal A, Morissette J, Menninger J, Lieman J, Desai T, Banks A, Bray-Ward P, Ward D, Hudson T, Gerety S, Foote S, Stein L, Page DC, Lander ES, Weissenbach J, LePaslier D, Cohen D (1995): A YAC contig map of the human genome. Nature **377** (Suppl): 175–297

Collignon J, Varlet I, Robertson EJ (1996): Relationship between asymmetric *nodal* expression and the direction of embryonic turning. Nature **381**: 155–158

Dib C, Fauré S, Fizames C, Samson D, Drouot N, Vignal A, Millasseau P, Marc S, Hazan J, Seboun E, Lathrop M, Gyapay G, Morissette J, Weissenbach J (1996): A comprehensive genetic map of the human genome based on 5,264 microsatellites. Nature **380**: 152–154

Fantes J, Redeker B, Breen M, Boyle S, Brown J, Fletcher J, Jones S, Bickmore W, Fukushima Y, Mannens M, Danes S, van Heyningen V, Hanson I (1995): Aniridia-associated cytogenetic rearrangements suggest that a position effect may cause the mutant phenotype. Hum Mol Genet **4**: 415–422

Fishman GI, Eddy RL, Shows TB, Rosenthal L, Leinwand LA (1991): The human connexin gene family of gap junction proteins: distinct chromosomal locations but similar structures. Genomics **10**: 250–256

Foster JW, Dominguez-Steglich MA, Guioli S, Kwok C, Weller PA, Stevanovic M, Weissenbach J, Mansour S, Young ID, Goodfellow PN, Brook JD, Schafer AJ (1994): Campomelic dysplasia and autosomal sex reversal caused by mutations in an *SRY*-related gene. Nature **372**: 525–530

Hudson TJ, Stein LD, Gerety SS, Ma J, Castle AB, Silva J, Slonim DK, Baptista R, Kruglyak L, Xu S-H, Hu X-T, Colbert AME, Rosenberg C, Reeve-Daly MP, Rozen S, Hui L, Wu X, Vestergaard C, Wilson KM, Bae JS, Maitra S, Ganiatsas S, Evans CA, DeAngelis MM, Ingalls KA, Nahf RW, Horton LT Jr, Anderson MO, Collymore AJ, Ye W, Kouyoumjian V, Zemsteva IS, Tam J, Devine R, Courtney DF, Renaud MT, Nguyen H, O'Connor TJ, Fizames C, Fauré S, Gyapay G, Dib C, Morissette J, Orlin JB, Birren BW, Goodman N, Weissenbach J, Hawkins TL, Foote S, Page DC, Lander ES (1995): An STS-based map of the human genome. Science **270**: 1945–1954 [with supplementary data from the Whitehead Institute/MIT Center for Genome Research, Human Genetic Mapping Project, Data Release 11 (October 1996)]

Kato R, Yamada Y, Niikawa N (1996): *De novo* balanced translocation (6;18)(q21;q21.3) in a patient with heterotaxia. Am J Med Genet **66**: 184–186

Kato R, Matsumoto N, Niikawa N (1997): Assignment of the human connexin43 gene, *GJA1*, to

chromosome 6q22.3. Jpn J Human Genet **42**: 213-216
Kuwano A, Mutirangura A, Dittrich B, Buiting K, Horsthemke B, Saitoh S, Niikawa N, Ledbetter SA, Greenberg F, Chinault AC, Ledbetter DH (1992): Molecular dissection of the Prader-Willi/Angelman syndrome region (15q11-13) by YAC cloning and FISH analysis. Hum Mol Genet **1**: 417-425
Levin M, Johnson RL, Stern CD, Kuehn M, Tabin C (1995): A molecular pathway determining left-right asymmetry in chick embryogenesis. Cell **82**: 803-814
Lowe LA, Supp DM, Sampath K, Yokoyama T, Wright CVE, Potter SS, Overbeek P, Kuehn M (1996): Conserved left-right asymmetry of nodal expression and alterations in murine *situs inversus*. Nature **381**: 158-161
Meno C, Saijoh Y, Fujii H, Ikeda M, Yokoyama T, Yokoyama M, Toyoda Y, Hamada H (1996): Left-right asymmetric expression of the TGF-β-family member *lefty* in mouse embryos. Nature **381**: 151-155
Naylor SL, Chin WW, Goodman HM, Lalley PA, Grzeschik K-H, Sakaguchi AY (1983): Chromosome assignment of the genes encoding the alpha and beta subunits of the glycoprotein hormones in man and mouse. Somatic Cell Genet **9**: 757-770
Ohta T, Tohma T, Soejima H, Fukushima Y, Nagai T, Yoshiura K, Jinno Y, Niikawa N (1993): The origin of cytogenetically unidentifiable chromosome abnormalities: six cases ascertained by targeted chromosome-band painting. Hum Genet **92**: 1-5
Roessler E, Belloni E, Gaudenz K, Jay P, Berta P, Scherer SW, Tsui L-C, Muenke M (1996): Mutations in the human *Sonic Hedgehog* gene cause holoprosencephaly. Nature Genet **14**: 357-360
Splitt PM, Burn J, Goodship J (1995): *Connexin43* mutations in sporadic and familial defects of laterality. N Engl J Med **333**: 941 only
Torgersen J (1949): Genic factors in visceral asymmetry and in the development and pathologic changes of lungs, heart and abdominal organs. Arch Pathol **47**: 566-593
Vortkamp A, Gessler M, Grzeschik K-H (1991): GLI3 zinc-finger gene interrupted by translocations in Greig syndrome families. Nature **352**: 539-540
White DM, Mikol DD, Espinosa R, Weimer B, Le Beau MM, Stefansson K (1992): Structure and chromosomal localization of the human gene for a brain form of prostaglandin D-2 synthase. J Biol Chem **267**: 23202-23208
Willecke K, Jungbluth S, Dahl E, Hennemann H, Heynkes R, Grzeschik K-H (1990): Six genes of the human connexin gene family coding for gap junctional proteins are assigned to four different human chromosomes. Eur J Cell Biol **53**: 275-280
Yancey SB, Biswal S, Revel J-P (1992): Spatial and temporal patterns of distribution of the gap junction protein connexin43 during mouse gastrulation and organogenesis. Development **114**: 203-212
Yokoyama T, Copeland NG, Jenkins NA, Montgomery CA, Elder FFB, Overbeek PA (1993): Reversal of left-right asymmetry: a situs inversus mutation. Science **260**: 679-682