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Association Of Nucleolus Organizer Regions Of Human Acrocentric Chromosomes.

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ASSOCIATION OF NUCLEOLUS ORGANIZER REGIONS OF HUMAN ACROCENTRIC CHROMOSOMES

by

Janice Leigh Smith

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Physiology and Biophysics in The Graduate School, University of Alabama in Birmingham

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ABSTRACT OF DISSERTATION

GRADUATE SCHOOL, UNIVERSITY OF ALABAMA IN BIRMINGHAM

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Title Association of Nucleolus Organizer Regions of Human Acrocentric Chromosomes

The stalk DNA of human acrocentric chromosomes codes for 18s + 28s rRNA; this enables the stalks to participate in nucleolus formation. Remnants of this function are seen on silver stained mitoses in the form of stalk associations in which two or more acrocentrics are connected at the stalks by a silver stained protein strand. It has been suggested that these connectives are strong enough to compete with the spindle apparatus and cause nondisjunction resulting in a trisomic cell line. In order to compile data which would support or reject the hypothesis, stalk associations in karyotypically normal individuals from the general population were compared with associations from karyotypically normal couples who had had a child with Trisomy 21.

Two parameters showed no differences as both groups had equal numbers of Ag-NORs per cell and per D and G group chromosomes. The size of association complexes varied little among individuals and between groups.

The average association rate of the parents was higher than that of the controls because of the parents' increased number of cells containing multiple association complexes. Neither difference was significant, but a significant increase in the average number of complexes per cell was observed in the parents.

Chromosomes associated either randomly or non-randomly depending on the individual. Among controls, a non-random pattern emerged as 22 and then 21 associated most frequently. A non-random pattern was observed in the parents as 21 was the most frequently associating chromosome in mothers and fathers. The increased frequency of 21 in the parents was highly significant; the frequency of the other acrocentrics was similar in both groups.

Non-random pairing of chromosomes was observed as 21-22 was the most common non-homologous pair and 21-21 the most frequent homologous pair in both groups. But, a significant increase in G-G pairs, and a highly significant increase in the total number of G-G and D-G pairs containing a 21 were observed in the parents.

The data support the theory that associations play a causal role in nondisjunction; the overlap in the range of values is too great to use any one parameter for high risk screening.

Abstract Approved by: Committee Chairman_____

Program Director_____

Date_____ Dean of Graduate School_____

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CHAPTER I

INTRODUCTION

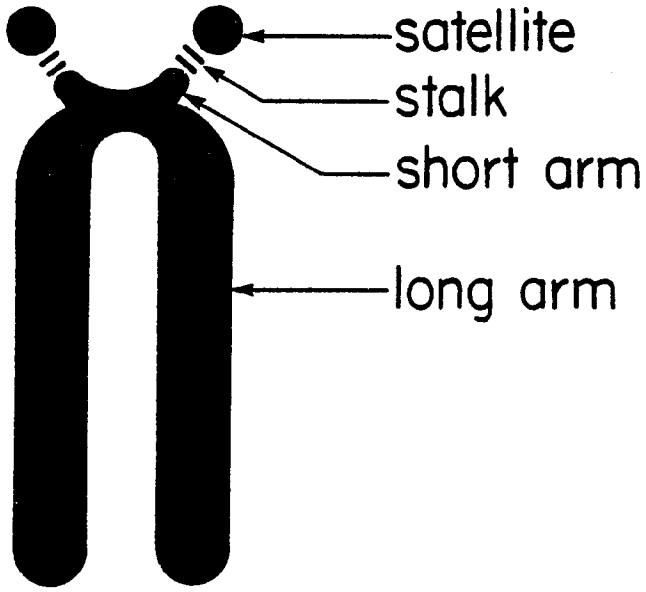
Nucleolus Organizer Regions were originally described by investigators studying the morphology of chromosomes in plants and lower animals. Differential staining of cells from the saffron plant Crocus sativus enabled Gates and Pathak (1938) to visualize a light green nucleolus in contrast to red chromatin; three of the chromosomes bore satellites connected to their long arms by thin threads which also stained a reddish color indicating chromatin. They were also able to see on favorably positioned chromosomes small green pronucleoli arising at the red threads and fusing into a single nucleolus of normal size. Therefore, this red staining thread became known as the Nucleolus Organizer Region or "NOR."

The study of human NORs had to await improvement of the in vitro culturing and harvesting of fibroblasts from various tissues in order that chromosomal number could be correctly determined and chromosome morphology more intensely studied. After Tjio and Levan (1956) published results of a study which indicated that the human diploid number of chromosomes per cell was 46, not 48 as previously thought, efforts were made to differentiate individual pairs of chromosomes on the basis of size and centromeric position as well

as the presence of satellites. Early investigations indicated that some of the five pairs of acrocentric chromosomes carried satellites (Figure 1) while others did not. There was, however, some confusion as to which chromosomes carried the satellites; this confusion was due partially to the different nomenclature classifications used by the various investigators (Tjio and Puck, 1958; Chu and Giles, 1959; Levan and Hsu, 1959). At the Denver Conference on nomenclature (1960), it was stated that the number 13 had prominent satellites while the number 14 had smaller ones and the number 15 had none. Of the two smallest pairs, the number 21 was assigned to the chromosome which bore satellites as it was concluded that the other pair and the morphologically similar Y did not bear satellites (Denver Conference, 1960).

Ferguson-Smith and Handmaker (1961) were the first to demonstrate satellites on all ten acrocentric chromosomes and to describe the phenomenon of satellite association. In 250 mitoses, no satellites were observed on any except the five pairs of acrocentric chromosomes, 28 cells were shown to have all six D group chromosomes bearing satellites, and 62 metaphase plates were observed to contain all four G group chromosomes with satellites. In 60% of the cells, two, three, or more of the acrocentric chromosomes were oriented in the spread so that their satellites or short arms were either in contact with or in very close proximity to one another. Ferguson-Smith and Handmaker called this phenomenon "satellite association" and proposed that this might be one of the

Figure 1 Schematic representation of the morphology of a human acrocentric chromosome.


satellite
stalk
short arm
long arm

determining factors in nondisjunction and centric fusion of chromosomes. They further stated that the adhesive property of the chromosomes was probably related to the role of the secondary constriction in nucleolus formation. In spite of this observation, research during the following decade would focus on the satellites, not the threads or stalks which bridged the satellites and the short arms.

Ohno et al. (1961) attempted to determine the number of chromosomes participating in the organization of the nucleolus by scanning prophase and late prophase mitoses for visual evidence of pronucleoli associating with the satellites and stalks of the ten acrocentrics. While the actual numbers were low, more recent accurate methods indicating participation in nucleolar formation have proved the accuracy of their conclusions. They suggested that not all, perhaps only five or six, of the ten NOR bearing chromosomes were needed for nucleolus organization; therefore, some NORs were functionally active and some inactive at any given time.

As the ease of obtaining and culturing lymphocytes instead of fibroblasts became apparent, research efforts in cytogenetics expanded and satellite association studies proceeded in several directions. Efforts were made to characterize the phenomenon as to the types and frequencies of association as well as the randomness or non-randomness of participation by each chromosome. To determine the degree of involvement of satellite association in chromosomal aberrations, association frequencies were examined in both chromosomally normal and abnormal populations. Investigators

studied age, sex, the size of the satellite-stalk-short arm region, and culture techniques in order to determine which factors might influence satellite association frequencies.

It was established that all acrocentrics (D group chromosomes 13, 14, and 15 and G group chromosomes 21 and 22) (see Figure 2) were at some time involved in satellite association both with their homologues and with non-homologous chromosomes (Bishun, 1966). At that time, it seemed that the greatest tendencies were for two chromosomes to form an association complex (Cohen and Shaw, 1967) and for there to be either one complex per cell (Frøland and Mikkelsen, 1964; Bishun, 1966) or two separate associations per cell with less than 10% of the cells devoid of satellite association (Cohen and Shaw, 1967; Zang and Back, 1968).

Whether chromosomes participate in association in a random or a non-random manner has been researched by various investigators over a period of twelve years without a definite answer having been reached. Since the first studies were without the advantage of banding, it was only possible to compare either the D:G ratio or the number of D-D and G-G combinations. The results seemed to indicate that the numbers 21 and 22 had an increased tendency to associate when compared with the three pairs of D group chromosomes. For example, an increase over the expected number of G-G pairs was observed; also seen was a D:G ratio of 1:1 when a 3:2 ratio was expected (Cohen and Shaw, 1967; Zang and Back, 1968; Galperin, 1969b).

Figure 2 A Karyotype from a normal human female cell depicting the division of the autosomes into seven groups lettered A-G. Banding techniques allowed further designation by number. (Some D and G group chromosomes also display silver deposits at the secondary constriction.)

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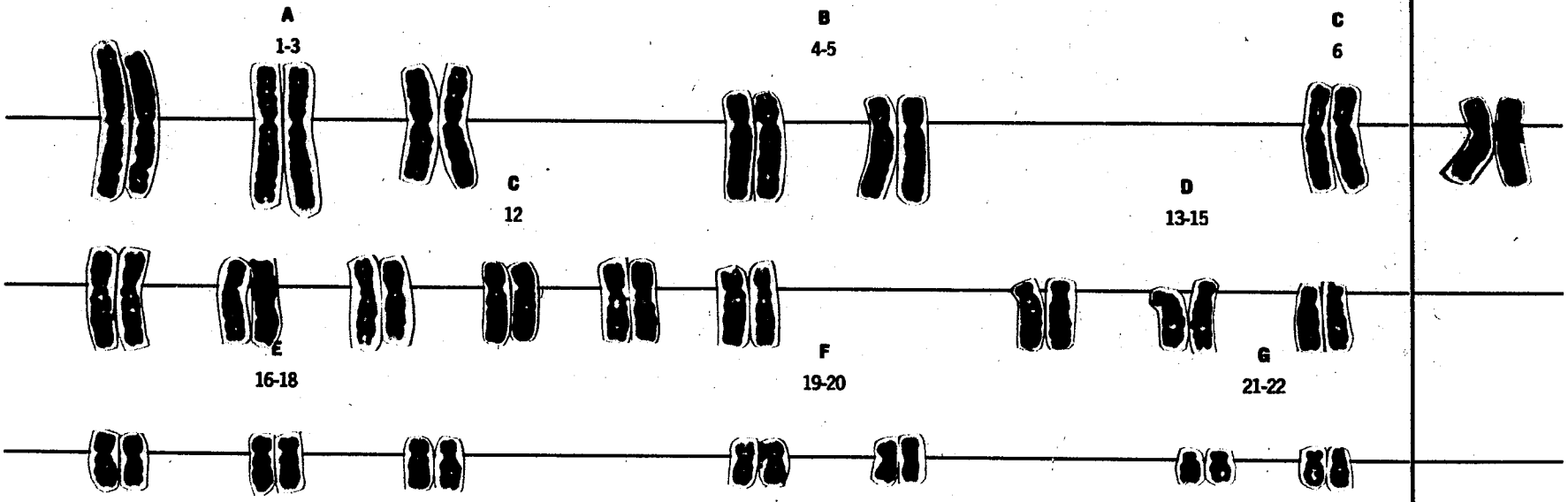


Bishun (1966) karyotyped the chromosomes of the D and G groups on the basis of size and noted a random distribution of numbers 13, 14, 15, and 22 among the association complexes but a highly increased association percentage for the number 21. Autoradiography studies partially supported his work; culturing cells in the presence of tritiated thymidine allows the Ds, but not the Gs, to label differently so that they can be distinguished from one another. Both Nakagome (1969) and Shaw et al. (1969) reported random association among the D group chromosomes, whereas Cooke (1971) observed a non-random pattern.

The use of quinacrine mustard or digestive enzymes to produce alternating lightly stained and darkly stained bands on the chromosomes (Caspersson et al., 1971; Seabright, 1971) gave cytogeneticists the ability to distinguish each pair in an objective manner. This, however, did not settle the question of random or non-random association of chromosomes. Curtis (1974) examined 900 metaphases from 30 normal persons and found each pair of acrocentric chromosomes associating randomly. Five other studies, also on normal populations, indicated a non-random pattern of association, although there was no agreement as to which chromosome was most often in association. The results of two studies implied that the non-randomness with which the chromosomes associated was related to or was possibly responsible for the non-randomness with which acrocentric chromosomes were found in trisomies and translocations. Noting that Trisomy 21 is as common as Trisomy 22 is rare, Galperin-Lemaître et al. (1977) presented a population with an

excess of 21-21 pairs as opposed to 22-22 associations. The most common homologue pairing of the D group acrocentrics was 13-13; that chromosome represents the most frequently occurring D trisomy. Regardless of the person studied, Patil and Lubs (1971) found that the chromosomes most often involved in Robertsonian translocations, the numbers 13, 14, and 21, were more likely to be involved in satellite associations than the 15 or 22. Overall, the populations studied by other investigators each displayed a non-randomness in association frequencies, but the frequency with which each acrocentric pair entered association proved to be an individual characteristic (Schmid and Krone, 1974; Jacobs et al., 1976; Ardito et al., 1978). The use of fluorescent polymorphisms to distinguish homologues enabled Schmid and Krone to demonstrate that a significant difference in association frequencies sometimes existed between the two chromosomes so that one of the pair could be almost totally responsible for the overall rate of association. It seems, therefore, that the tendency to associate is a heritable characteristic of an individual chromosome and that the association value for a given pair is a function of the independent frequencies of both homologues. The theory that association tendencies are heritable was supported by Zang and Back (1968) who observed a consistency in association values from sample to sample in the same person but a lack of consistency from person to person.

A test of randomness versus non-randomness was also applied to the question of whether chromosomes have a preference as to association partners. Again, the results were inconsistent from author

to author. Jacobs et al. (1976) saw a non-random pattern of pairings in each individual, but when all results were combined, it randomized the pairings in the population so that one chromosome seemed to associate with any other at an approximately equal rate. Both Ardito et al. (1978) and Galperin-Lemaître et al. (1977) noted a significant excess of pairs involving the number 13 as well as the number 21 in the population as a whole.

From a review of both clinical and cytogenetic literature, it seems that acrocentric chromosomes are extremely vulnerable to nondisjunction and rearrangements, possibly more so than the other more metacentric chromosomes. Since the acrocentrics are the sites of the nucleolus organizers, scientists hypothesized that the vulnerability was related to satellite association, a remnant of nucleolus organization, and began examining association values in chromosomally normal and abnormal populations. Trisomy 21 (Down's Syndrome) has been most often studied since it is the most common autosomal disorder in man.

Before banding procedures were available, several investigators simply examined the number of satellite associations per 100 cells in mongoloid children, their parents, and control individuals. Luchsinger et al. (1969) recorded a significant increase in association frequency in the children and their parents over that in the controls. Rosenkranz, however, only found a significant increase in the patients (Rosenkranz and Fleck, 1969), not in their parents (Rosenkranz and Holzer, 1972).

With banding came the ability to look at specific chromosomes, especially the number 21 which is important in the causation of Down's Syndrome. Unfortunately, results from various studies have not always conformed with one another. One point of agreement, however, was the fact that the number 21 associated very frequently regardless of the population under study. In fact, two studies found no significant difference in the association frequency of the 21st chromosome pair between parents of afflicted children and controls matched for age and sex. They did find the number 21 most often associating in both groups to a significant degree. They also found a non-random pattern of pairing, but the most common pairs, which usually included the 21, remained fairly consistent from group to group (Taysi, 1975; Davison et al., 1981). Unlike Luchsinger's and Rosenkranz's results, the number of associations per cell in Davison's study did not vary among the children, their parents, or their controls. Since the number 21 associated at approximately the same rate in the control and parent groups, Taysi believed that there was no real relationship between the frequency of satellite association of the number 21 and its frequent participation in nondisjunction. A significant increase in the association values of the 21 in parents as opposed to controls was noted by Curtis (1974) and Hansson (1979). In Curtis' families, both the fathers and mothers showed a significantly higher association rate, whereas Hansson usually observed the highest values in the mothers. This proved to be especially true in those women who had had more than one child with Down's Syndrome and in those between the ages

of 20 and 29. Some extremely high values were found in several fathers indicating possible sources of paternal nondisjunction.

Results of studies on satellite association have, at best, been diverse and often completely contradictory. Possible explanations for this difference include the criteria for defining an association; many have relied on the definition stated by Cohen and Shaw (1967), whereas others have defined their own limits. Most have stated that the short arms of the acrocentrics must be oriented toward each other, but the allowable distances between them have ranged from the length of Gq (Cohen and Shaw, 1967) to the width of a single chromatid (Rosenkranz and Holzer, 1972). Galperin even used computer aided measurements to determine if an association existed (Galperin, 1969a).

A major source of variation in both the quantitative and qualitative assessments derived from satellite association studies could reside in the different culturing and harvesting techniques employed. Chromosomes may be derived from macroculture techniques such as the one of Moorhead et al. (1960) in which a leukocyte-plasma suspension is added to the medium or by microculture techniques such as the one developed by Arakaki and Sparkes (1963) which uses whole blood. Using the same medium for both techniques, Hansson (1970) noted, in the microcultures, a significant increase in such quantitative parameters as the number of cells containing an association and the number of acrocentrics which participated in association; when D:G ratios were compared, no qualitative differences between the two methods were evident. Zang and Back (1968)

also saw differences in association values for the two methodologies, but their quantitative increase appeared in the macrocultures. Both techniques showed an increase in G-G associations over expected values while showing a decrease in the number of D-D pairs. Since that study used a different medium for each procedure, a subsequent study (Back and Zang, 1969) was done using the same medium in each culture. No quantitative differences were noted but the microcultures appeared to have significantly more G-G and fewer D-D associations than macrocultures.

Varying results might also be attributed to different culture conditions. Back and Zang (1969) showed no quantitative and very little qualitative association differences between TC 199 and McCoys 5A cell culture medium. Both an increased glucose concentration in the medium and an increase in the incubation temperature have been shown to decrease satellite association frequencies (Hoehn et al., 1971). Nankin (1970) has demonstrated an inverse effect of the length of time cells remain in culture on the qualitative measurements of association. The number of cells containing an association complex and the number of chromosomes per complex steadily declined as the culture remained in incubation from 48 to 72 to 96 hours. The fact that fibroblasts are carried in culture for a much longer period of time than lymphocytes might explain the sometimes significant decrease in the frequency of satellite associations in fibroblasts as compared to lymphocytes from the same person (Frøland and Mikkelsen, 1964; Higurashi and Conen, 1971).

Other possibilities to explain the variant results often found in association studies include the amount of acetic acid in fixative; in an extreme demonstration, Back and Zang (1969) resuspended some cells in 70% acetic acid and saw a significant rise in association values. They also seemed to believe that the more vigorous pre-cleaning of slides which would allow chromosomes to spread better would decrease satellite association. Still others have concluded that it is the individual's unique chromosome complement which represents the main source of variation (Jacobs et al., 1976).

It is important to recognize that genetic heterogeneity of an individual should be taken into account when data are collected from a number of different persons and then analyzed together as one. Cohen and Shaw (1967) recognized this as a possible factor contributing to variable results in association studies as did others who investigated differences in male and female cells. Zang and Back (1968) could find no significant differences in the number of cells containing at least one association complex, in the number of complexes per cell, or in the types of associations when they compared these parameters in mitoses from male and female cells. Work by Ardito et al. (1978) and Frøland and Mikkelsen (1964) supported the hypothesis that no significant differences exist between male and female cells in regard to association values. Galperin (1969a), however, noted a general increase in the frequency of associations in female cells over that in male mitoses. Similar results were noted on a later study in which chromosome

banding gave some additional information; chromosome involvement in association in males seemed to be generally random, whereas the tendency for 21 and 13 to associate significantly more often in female cells contributed to a non-random association pattern. This could perhaps be taken as a partial explanation for the more frequent maternal contribution of the supernumerary chromosome in both Trisomy 21 and Trisomy 13 (Galperin-Lemaître et al., 1980).

Any relationship which might exist between the age of a subject and the frequency of satellite association is somewhat obscured by all the conflicting results. The most recent data indicate that there is a fluctuation in association values as an individual ages. Low association frequencies have been commonly observed in newborns and young children, but were seen to increase as a child entered his teenage years. In the third decade of life, however, a drop in the frequency of associations has been noted; this decrease tended to level off and, in fact, to reverse itself after 30 years of age. Association values in the elderly have been seen to decrease (Hansson, 1979).

It is well known that the region of the acrocentric chromosome which includes the short arm, stalk, and satellite, is often structurally polymorphic from chromosome to chromosome without any clinical significance to an individual (Zankl and Zang, 1974). With the use of fluorescence, Schmid and Krone (1974) noticed that a particular chromosome remained morphologically similar from one mitosis to the next; in other words, chromosomes either did or did not consistently possess fluorescent satellites. The length of the

secondary constriction also seemed to be a consistent feature as it was faithfully replicated with the rest of the chromatin. They found that the homologue with the longer stalks was more likely to participate in association than its partner with shorter constrictions. Orye (1974) agreed that association values increased with increasing length to a certain point; extremely long secondary constrictions were found to have a decreased association frequency. It was concluded that since satellite association was probably a function of nucleolus formation where enough copies of rRNA genes were collected in close proximity to form a nucleolus, those acrocentrics with very long stalks possessed enough gene copies to produce a nucleolus alone. Some polymorphisms such as sitting satellites, deleted satellites, deleted or exceptionally large short arms have been seen on chromosomes with reduced association frequencies (Orye, 1974; Schmid and Krone, 1974; Zankl and Zang, 1974) while enlarged and double satellites seemed to enhance the tendency of the chromosome to associate (de Capoa et al., 1973; Zankl and Zang, 1974).

While some investigators continued to research satellite association, others became interested in what is encoded in the genes of the region. The discovery of a silver stain for the NORs and the discovery of the nature of the substance being stained introduced new areas of research.

The study of human NORs was advanced considerably by the use of the in situ hybridization and autoradiographic techniques. Tritium labeled 18s + 28s rRNA was isolated from fibroblasts grown

in the presence of [^{3}H] uridine. Slides, which had been made from lymphocyte cultures established and harvested in the conventional manner, were incubated with a solution containing the labeled RNA. Coating the slides with a photographic emulsion allowed grain counts to be made on metaphase plates. The number of grains was considered a function of the amount of rRNA hybridized to the chromosomes which in turn indicated the location and amount of rDNA, the DNA which codes for 18s + 28s rRNA (Henderson et al., 1972). These studies also revealed the heteromorphic characteristics of NORs as well as possible new influences on satellite association.

Two observations, the association between the nucleolus and the satellite region of the acrocentric chromosomes during interphase as well as the participation of these chromosomes in satellite association seen during metaphase (Ferguson-Smith and Handmaker, 1961; Ohno et al., 1961), were considered indirect evidence that genes coding for some ribosomal subunits of the nucleolus were located in or near the satellites. The in situ hybridization studies did indeed demonstrate exclusive localization of 18s + 28s rRNA cistrons to the satellite region of chromosome numbers 13, 14, 15, 21, and 22; no other chromosomes were specifically labeled whereas all ten acrocentrics hybridized some rRNA. Furthermore, no significant differences in grain counts were found between D and G group chromosomes (Henderson et al., 1972; Evans et al., 1974).

Evans and his colleagues (1974) utilized the heteromorphic characteristics of the satellite region to establish that the exact location of the 18s + 28s rRNA genes was the stalk, not the satellites or the short arms. In situ hybridization studies were done on the chromosomes of an individual whose number 15s differed considerably in regard to the length of the stalks while the size of the satellites was approximately equal. The homologue bearing the longer stalk hybridized four times the amount of tritium labeled rRNA as did its partner. When the experiment was repeated on homologues heteromorphic for satellite size but with stalks of equal length, the grain counts on both chromosomes were equivalent. They therefore concluded that the stalk alone was the site of the rRNA cistrons.

The fluorescent polymorphisms of the short arms, stalks, and satellites have been well established as has the stability of these polymorphisms as a chromosome is passed from parent to progeny. The various in situ hybridization studies have indicated that the number of rRNA genes encoded in the DNA of the stalk is also a heteromorphic characteristic of this region; variations in the number of gene copies were seen between chromosome pairs as well as between homologues. Because this difference is also a heritable consistent feature of a particular chromosome, investigators concluded that individuals might differ in the total amount of rRNA contained in their genome; this of course would be a function of the number of gene copies on each acrocentric inherited from both parents (Evans et al., 1974; Dittes et al., 1975; Warburton et al.,

1976). Warburton et al. (1976) provided proof for this conclusion when they compared totaled grain counts from all acrocentrics and found significant variation among individuals.

Just as no consistent correlation has been demonstrated between fluorescent and in situ hybridization heteromorphisms, the experimental data will not allow a simple correlation to be made between the association frequency of an acrocentric and the number of rRNA gene copies on its stalk. The exception is that the presence of rDNA was deemed necessary for the formation of association complexes since these complexes are considered to be remnants of nucleolus formation which occurs during interphase (Evans et al., 1974; Henderson and Atwood, 1976; Warburton et al., 1976). In 1973, Henderson et al. had sometimes observed silver grains connecting the satellite regions whether or not a thread was visible with a routine staining procedure. When this occurred between chromosomes separated by some distance, the number of grains in the longer connective threads was approximately equal to the number of grains observed over chromosomes very closely associated. They therefore agreed that the two "types" of associations represented the same phenomenon. From this piece of evidence, they concluded that association complexes are a remnant of the transcription of rRNA sequences in order to form a nucleolus. During transcription, the DNA of two or more chromosomes would be closely associated, sometimes becoming entangled. This entanglement could possibly persist beyond interphase to be observed during metaphase as satellite association first described by Ferguson-Smith and Handmaker

(1961). It was then proposed that the rDNA content influences the frequency of connectives and therefore the association frequency, so that in a person whose chromosomes appear to associate randomly, all ten acrocentrics would have a similar number of gene copies. The rDNA, however, would be disproportionately distributed among the ten stalks in an individual with a non-random association pattern.

Because of the following evidence, investigators agreed that other factors besides rDNA content must take part in determining the association frequency for a particular chromosome. Early evidence with grain counts, which showed that the number of grains over associated chromosomes did not seem to be any more abundant than the grains over non-associated chromosomes, indicated that chromosomes with similar amounts of rDNA had different association frequencies (Henderson et al., 1972; Evans et al., 1974). In support of this was the previously discussed individual studied by Evans et al. (1974) whose 21st and 22nd pairs had equal grain counts but the 21s were found in association more often than the 22s. His marker 15 with the very high grain count was rarely in association. A couple of years later, cases were reported which supported the theory that an increased number of rRNA genes tended to increase the association frequency (Henderson and Atwood, 1976; Warburton et al., 1976). Henderson's case involved a double satellited 15 which hybridized five times as much [^{3}H] rRNA as the other chromosomes and was found in association more often than all other chromosomes. They acknowledged the possibility that the increased

number of satellites or another unknown factor could be at least partially responsible for the increased association rate.

The discovery that the connective threads bridging the satellited ends of acrocentric chromosomes probably contained the same nucleic acid as the NORs themselves emphasized the possible importance of satellite association in the causation of chromosomal anomalies. Since in situ hybridization was a tedious technique which required a certain amount of expertise to perform, thorough study of the region was still hindered by the lack of a simple procedure which differentially and definitively stained the NORs. In addition, conventional staining methodology often left investigators arbitrarily guessing as to what was actually an association.

The extraction of nucleic acids and histones followed by Giemsa staining produced N bands which most often appeared at the secondary constrictions of the acrocentrics and in the nucleoli of interphase nuclei (Matsui and Sasaki, 1973). Other laboratories could not satisfactorily reproduce their results, however, and attention turned to a modification of an ammoniacal silver procedure used previously on chromosome preparations of lower animals. This four step procedure known as the Ammoniacal Silver-Satellite (AS-SAT) technique differentially stained the satellite regions and any physical connectives which might exist between the chromosomes (Howell et al., 1975). Goodpasture and Bloom (1975) simplified the procedure with a new two step technique which they designated Ag-AS for the silver and ammoniacal silver solutions employed. These two

time consuming methods were not without their problems, however. It was impossible to standardize the staining times because of the extreme instability of the solutions. Although the staining procedure was monitored with a microscope, uneven staining across the slide usually occurred, resulting in inaccurate silver positive NOR (Ag-NOR) counts. Precipitation of silver grains on the slide often set up a mirror-like background which interfered with the study of the chromosomes. Howell and Black (1980) developed a simple, time efficient, one step process which eliminated many of the problems of the earlier methods. For the first time, the silver staining technique was standardized and required no monitoring with a microscope while obtaining reproducible results from slide to slide. While the background remained clean, metaphase plates on opposite ends of the slide were evenly stained thus enabling the investigator to obtain reliable Ag-NOR counts.

Although any of the three silver methodologies stained the 18s + 28s rRNA gene loci, experiments indicated that it was highly unlikely that the silver was being incorporated into the DNA, itself; DNase and RNase pretreatment of the chromosome spreads did not eradicate the Ag-NORs. On the other hand, exposure of slides to proteolytic enzymes, such as pronase and trypsin, prior to silver staining did eliminate any silver staining of the NORs. This, coupled with the fact that the secondary constriction regions proved to be insoluble in acid but slowly soluble in a dilute alkaline solution, implied that it was acid nucleoproteins which

were being stained (Goodpasture and Bloom, 1975; Howell et al., 1975).

As the in situ hybridization experiments showed individual variability of the NORs, so did the staining of the region with $AgNO_3$. The size of an NOR, as determined by the amount of silver deposited on the nucleoprotein, varied not only among all ten acrocentric chromosomes but also between homologues; this is in exact agreement with the in situ experiments. The number and the identity of chromosomes per cell bearing Ag-NORs was found to remain fairly constant from cell to cell within an individual but was found to vary from one person to another in a sample population (Goodpasture and Bloom, 1975; Howell et al., 1975; Goodpasture et al., 1976; Mikelsaar et al., 1977a). While all ten acrocentrics were stained by in situ hybridization indicating gene copies on each D and G group chromosome, most cells did not contain ten Ag-NORs. Two theories were advanced to explain this phenomenon: either the rRNA cistrons represented an unstable system in which many gene copies were regularly deleted or some genes were being inactivated in the majority of cells because there were more present than necessary to form a normal nucleolus (Evans et al., 1974; Miller et al., 1976a).

Miller et al. (1976a; 1976b) found support for the inactivation theory in silver staining experiments on mouse-human hybrid cell lines, some of which preferentially lost human chromosomes while others preferentially lost mouse chromosomes. In those lines

losing human chromosomes, only mouse rRNA, not human rRNA, was detected in the cytoplasm of the cell. In hybrid cells which contained some human acrocentric chromosomes, Ag-NORs were found only on mouse chromosomes even though the human chromosomes were silver positive in the parent line. In the hybrid lines which lost mouse chromosomes, the cytoplasm contained human rRNA, and the $AgNO_3$ stained only human NORs in spite of the fact that mouse NOR bearing chromosomes which had been active in their parent line were still present in the hybrid cells. They concluded that only the rRNA genes which had been transcribed during the previous interphase showed positive staining with $AgNO_3$, thus implying that in a human cell line, only a portion of the genome was usually necessary for nucleolus formation. Furthermore, since the silver staining pattern of a chromosome seemed to be consistent from cell to cell, the activity or inactivity of the genome in a particular chromosome must be an inherent characteristic.

In spite of the fact that Evans et al. (1974) had shown the stalk to be the site of the 18s + 28s rRNA genes with in situ hybridization and in spite of the fact that both the AS-SAT and Ag-AS procedures seemed to stain exclusively the same region, there was still some disagreement as to whether the silver was being deposited on the satellites or in the stalks. In many metaphase spreads, the stalks were so contracted as to be almost indistinguishable from the satellites; this was especially true after silver staining when the silver grains often obliterated all of the short arm, stalk and satellite. In much the same manner as Evans,

Goodpasture et al. (1976) used late prophase and prometaphase chromosomes which had elongated stalks to demonstrate that the short arms of the chromosomes were distinctly situated below the silver stained NORs while the satellites were clearly above them. It is now widely accepted that the stalks code for 18s + 28s rRNA and that it is this structure which, if actively transcribed, can be preferentially stained with an $AgNO_3$ solution.

Research designed to characterize Ag-NORs was similar to that designed to elucidate the characteristics of satellite association. In fact, a major point of interest was the relationship between satellite association and Ag-NORs. Miller et al. (1977) reasoned that since both were supposed to be an indication of participation in nucleolus formation, there should exist a positive correlation between both the presence and amount of silver stained material and the number of times the chromosome is involved in association. This is indeed what they, as well as others (de Capoa et al., 1978; Galperin-Lemaître et al., 1980; Hens et al., 1980), have found. One discrepancy did exist between the results of Miller and de Capoa regarding chromosomes bearing silver negative, or non-transcribed, NORs; de Capoa never found these in association whereas Miller did, although they were observed ten times less often than NORs stained by even the smallest amount of $AgNO_3$. It was concluded, therefore, that the frequency of satellite association is positively correlated with the presence and amount of silver stain which is a measure of the rRNA gene activity, not the number of genes present.

Experimental evidence supports the hypothesis that the distribution of silver positive and negative NORs is consistent in an individual, with most people having somewhere between 8 and 10 Ag-NORs per cell (Mikelsaar et al., 1977a) or 7 to 9 (Howell, pers. comm.). The occurrence of Ag-NORs on a particular chromosome seemed to be a characteristic of that chromosome (Mikelsaar et al., 1977a). Furthermore, a chromosome usually remained Ag negative from one mitosis to the next (Miller et al., 1977). The reason behind this, as Buys et al. (1979) have stated, is that the presence and size of the Ag-NOR is conserved after DNA replication; in other words, the sister chromatids have Ag-NORs of the same size. Mikelsaar et al. (1977a) did find a non-randomness, which was not significant, in regard to the distribution of Ag-NORs; a slight decrease in the frequency of Ag-NORs on chromosome numbers 14 and 22 was noticed.

The occurrence of Ag-NORs on a particular chromosome proved not only to be consistent within the individual but also as the chromosome is passed from generation to generation. Studying the chromosomes from seven children with Trisomy 21 and from their parents, Mikelsaar et al. (1977b) showed that the tendency for an NOR to be Ag positive or Ag negative remained consistent as the chromosome was passed from parent to child. In order to determine if a similar morphology was retained, Markovic et al. (1978) scored the size of the NORs on each acrocentric in silver stained and G-banded metaphase spreads of six patients and their parents. Upon determining an average NOR score for each chromosome, they found a

strong correlation between the two scores in the parents and their child. They concluded therefore that the amount of $AgNO_3$ taken up by an active NOR was an inherent characteristic of that NOR which could be passed from one generation to the next in a Mendelian fashion.

Several factors have been identified which may or may not enter into the determination of the modal number and distribution of Ag-NORs. The sex of an individual has not been seen to influence the frequency of Ag-NORs or their distribution among the ten acrocentrics (Mikelsaar et al., 1977a). Buys et al. (1979) examined mitoses from the very young and the elderly to investigate any inactivation of rRNA genes during the aging process. No significant difference was discovered in the total number of Ag-NORs per group, but the elderly did seem to have a decreased number of cells in which all ten NORs had actively participated in nucleolus formation. The health of an individual has been implicated in the causation of varying Ag-NOR frequencies and morphology. The mean number of NORs per cell seemed to be increased in cells from adenocarcinoma patients as opposed to the cells from normal controls, but only for G group chromosomes. No structural dissimilarities were noted (Cheng et al., 1981). Zankl et al. (1980) found the number 22 was more intensely stained after antithyroid treatment of hyperthyroid patients. A large source of variation may be the artificial stimulation of cells to synthesize rRNA by phytohemagglutinin (PHA); it is not yet known if PHA stimulates rRNA genes on the various chromosomes at the same rate or if it even activates

all the genes *in vitro* which were actively transcribed *in vivo* (Cheng *et al.*, 1981).

A positive correlation has been demonstrated between satellite association and silver staining of NORs which indicates stalk association. Satellite and stalk associations are probably the same phenomenon, but Ag negative acrocentrics have occasionally been observed in a metaphase plate with their short arms oriented toward one another in close proximity; this is the classical definition of satellite association. They were not in stalk association, however, since there was no silver stained protein bridge between them. This orientation could very possibly be due to random placement of the chromosomes in the metaphase spread. It is known that colcemid, an antimitotic agent, will disrupt the non-random spatial relationships dependent on the spindle fibers; the effect is much more pronounced on all chromosomes other than the acrocentrics, presumably due to their participation in association complexes (Rodman *et al.*, 1980). In other words, colcemid added to the culture medium randomizes placement in the metaphase plate of all chromosomes except for those acrocentrics whose NORs are connected via a protein bridge. Those acrocentrics not connected to one another will be randomly placed, and therefore, have as equal a chance of falling near a D or G group chromosome as they do of falling next to an A or C group chromosome.

It can be hypothesized that the protein bridges would be strong enough to compete with the spindle apparatus and cause nondisjunction; only those chromosomes whose NORs are bridged by

silver stained nucleoprotein and are therefore in stalk association should be counted when studying patients with a D or G trisomy and their parents. This criterion, which would remove any subjectivity from determining an association complex, would exclude not only Ag negative NORs in satellite association but also those Ag-NORs lying in close proximity, although not actually connected. Those chromosomes were probably associated at one time and separated either naturally before metaphase or during the harvesting or slide making technique. Some complexes appear to be more tightly bound than others. The weaker ones such as those broken during a vigorous harvesting procedure would probably be broken by the spindle. Those stronger ones which survive to appear in a metaphase spread might be able to compete with the spindle apparatus in anaphase and form chromosomally abnormal daughter cells if the connected chromosomes were supposed to segregate to opposite poles. This supposition is not completely without experimental support. Bobrow and Heritage (1980) used bromodeoxyuridine (BRdU) treated chromosomes to trace association pairs through three cell cycles seemingly without separating; each pair appeared to be acting as one chromosome on the spindle.

In order to determine if any evidence exists for the hypothesis that stalk associations play a causal role in nondisjunction, the general population as well as parents of children with a G or D trisomy should be evaluated. Chromosome number 21 has been shown in numerous studies to be commonly involved in satellite association and this may also prove true for stalk associations. Several

parameters may be studied for evidence that associations and non-disjunction are correlated. It was hoped that one or more of these parameters might lend itself to a screening test for people at an increased risk for having a child with a trisomy.

CHAPTER II

MATERIALS AND METHODS

In order to characterize stalk associations and to assess their relationship with chromosomal nondisjunction, venous blood (10 ml) was obtained from healthy individuals in the general population and from couples under 40 years of age who had had a child with Down's Syndrome. The control group consisted of nine males and nine females from 23 to 34 years of age. After being placed in sterile centrifuge tubes containing .5 ml of sodium heparin (Riker Labs), the blood was allowed to stand at room temperature for 1-2 hours until the plasma-leukocyte layer had separated from the red cell layer.

The white cells were cultured according to a modification of the method of Moorhead et al. (1960). Culture medium was made by dissolving lyophilized chromosome medium 1A (Grand Island Biological Company, Gibco) in 5 ml of diluent (Gibco). After transferring the medium to sterile culture flasks, 20 drops of the plasma-leukocyte suspension were pipetted into the medium and then incubated at 37°C for 72 hours.

One hour before harvesting the cultures, .2 ml of colcemid (10 µg/ml culture medium) (Gibco) were added to each flask to

arrest cell division in metaphase. After the cultures had incubated the final hour in colcemid, the contents of each flask were transferred to test tubes and centrifuged at 1000 RPM for 5 minutes. The medium was decanted and the cell pellet resuspended by tapping the tube. In order for the cells to swell, they were left in a hypotonic solution of .075 M KCl (4 ml) at 37°C for 10 minutes. After 1 ml of Carnoy's fixative (three parts methanol to one part glacial acetic acid) was added to the hypotonic-cell suspension, the tubes remained in the incubator for an additional 5 minutes. The tubes were again centrifuged at 1000 RPM, the supernatant discarded and the cell pellet resuspended. The cells were refrigerated in 4 ml of fresh fixative for a period of 12-24 hours before preparing slides.

Slides were first immersed in 95% ethanol, wiped dry, and then chilled in ice cold deionized water. The harvested cells in each tube were washed twice with fresh fixative to disperse as much of the cytoplasm as possible. After the final centrifugation, approximately .5 ml of fixative was added to the resuspended cells; three drops of this cell suspension were dropped onto a tilted slide from a Pasteur pipette. The slides were allowed to air dry and were then stored in slide boxes with desiccator capsules (Driaire) for at least a week before silver staining and banding techniques were performed.

The silver staining technique of Howell and Black (1980) was used to stain the active nucleolus organizer regions (NORs) and to visualize the associations between acrocentric chromosomes; it was

necessary to silver stain slides before banding since the trypsin would digest the protein to which the silver binds. This technique utilized two solutions which were used for no longer than one week and were then discarded. The silver solution consisted of 4 g $AgNO_3$ crystals (Fischer) dissolved in 8 ml of deionized water. The colloidal developing solution was composed of 1 g of gelatin, U.S.P. (J.T. Baker) in 50 ml of deionized water and 1 ml of formic acid (88%). Two drops of the colloidal developing solution and 4 drops of the silver solution were placed on the center of the slide and stirred with the edge of a coverslip which was subsequently applied to the slide. The slide was then placed on a slide warmer stabilized at 68°C for 90-120 seconds. Running deionized water was used to wash the coverslip and solutions from the slide. The NORs appeared as black dots while the chromosome arms had turned a golden yellow.

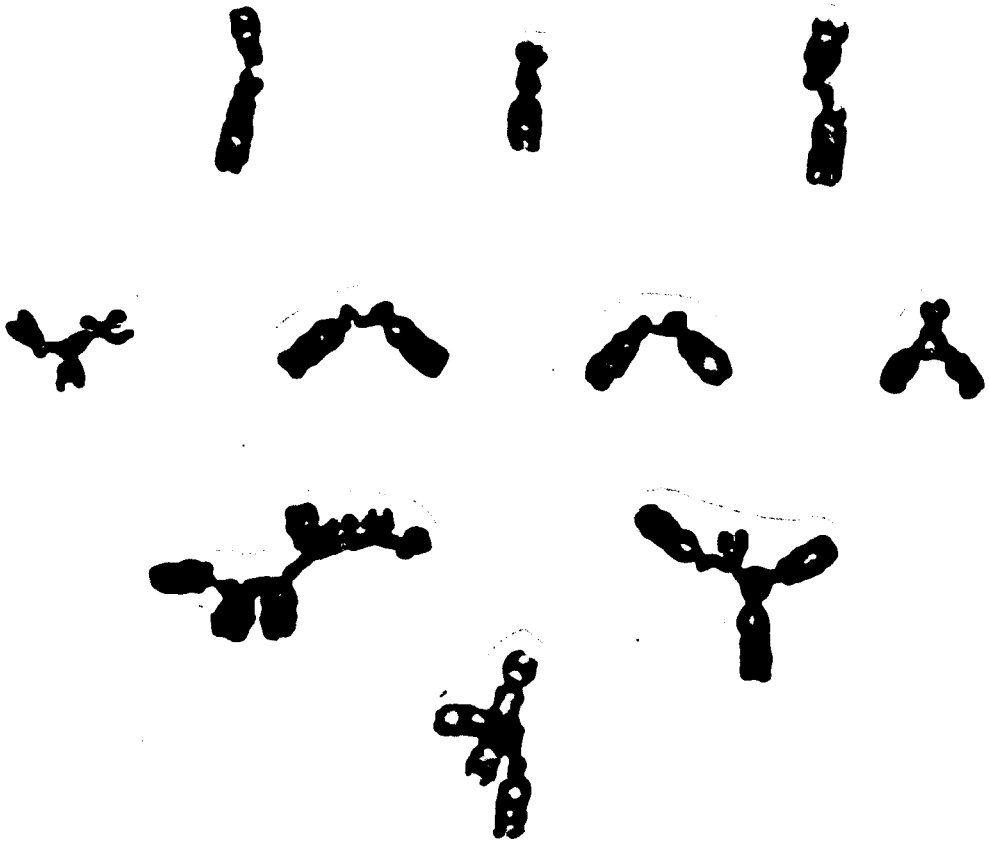
In order to correctly identify each chromosome bearing silver positive NORs and NORs participating in associations, the slides were banded by a modification of the method of Seabright (1971). Three coplin jars were filled with 42 ml of Hank's Balanced Salt Solution which had been adjusted to a pH of 8.1 by the addition of sodium phosphate dibasic, Na_2HPO_4 anhydrous (Merck). Three ml of a trypsin solution (2.5 g trypsin, Difco 1:250, in 100 ml of .9% NaCl) and 3 ml of an ethylenedinitrilotetraacetic acid solution (.5 g EDTA in 100 ml of .9% NaCl) were placed in the first jar. The slides were treated in the first jar for several seconds and rinsed in the second and third jars to stop the action of the

trypsin. To stain the chromosomes, the slides were placed for 5 minutes in a coplin jar containing 5 ml of Gurr R66 Giemsa in 37 ml of Sorenson's phosphate buffer (pH 6.8). The slides were put through two rinses of the buffer, mounted with a coverslip and viewed through a microscope.

The chromosomes of 50 metaphase plates from each subject were sketched at the microscope; the acrocentric chromosomes were identified and those bearing silver positive NORs were indicated on the sketch. The number of chromosomes in association (stalk association) was counted and the morphology of each association complex was noted. Two or more chromosomes were considered to be in association if their stalks were physically connected by silver grains. If chromosomes with silver positive NORs were lying in close proximity but no silver grains could be detected bridging the stalk regions, then these chromosomes were not considered to be in association. No association complex was composed of one chromosome with silver positive NORs and one with silver negative NORs even if their stalks appeared to be in very close proximity. Chromosomes, however, separated by any distance were recorded as being in association if silver grains bridged the NORs, even when this bridge was across another chromosome (Figure 3). Photographs were taken of representative spreads using Kodak technical pan 2415 film; however, all determination of associations was made with the microscope. Data were then compiled on each subject and combined with the appropriate group for statistical analysis.

Figure 3. Differentiation of associated and non-associated chromosomes by silver staining.

CHROMOSOMES NOT IN ASSOCIATION



CHROMOSOMES IN ASSOCIATION

CHAPTER III

RESULTS AND DISCUSSION

Data on the general association rate, the association frequencies of specific chromosomes and specific chromosome pairs, the number of association complexes per cell, the number of chromosomes per complex, and the frequency of Ag-NORs were compiled from observations of 50 metaphase spreads per individual studied. They are listed in the appropriate tables on the following pages.

A thorough study of stalk associations had two aims, one of basic research and the other of clinical importance. Comparison of the general association rates and the NOR mean and modal numbers from this study with previous ones would determine if populations from different geographical areas were homogeneous or heterogeneous. Evaluation of these and other parameters, such as the distribution of chromosomes in the association complexes and the average number of complexes per cell, should establish some baseline data with which to compare future results. Since it has been hypothesized that stalk associations might contribute to chromosomal nondisjunction, chi-square scores were calculated to determine if there were any significant deviations from expected chromosome behavior which might support the theory. Clinically, it would be

advantageous if a screening test were available to identify people at an increased risk for having a child with a trisomy before the birth of an affected infant. Unpaired t tests were run on mean scores to determine if any significant differences existed between a control population and a population of parents of children with Trisomy 21. If so, this would support the above theory and possibly present a screening test.

Tables I and II list the age and sex of each individual in the control population and the ages of the mothers and fathers of children with Trisomy 21. Trisomy 21 was chosen since it is the most common autosomal disorder in man. The maternal age effect in Down's Syndrome is well recognized, especially when the pregnant woman is at least 40 years of age. Women over the age of 35-37 also seem to be at some increased risk of having a child with Trisomy 21, but it is not nearly as pronounced at 35 as it is at 40. Most of these women are now given the option of amniocentesis for prenatal chromosome studies, but due to the lack of laboratories to process the amniotic fluid and due to the slight risk of the procedure, amniocentesis is not usually recommended for younger couples until after the birth of an affected child. Therefore, this study was confined to parents under 40 years of age where a screen for high risk couples would be more beneficial.

Efforts were made to keep the control population as homogeneous as possible yet obtain enough values from both males and females in several age groups to make the comparison with the parent group a valid one. Howell et al. (unpublished data) found no

TABLE I

CONTROL POPULATION*

Control	Sex	Age
L.A.	Female	24
A.B.	Female	29
D.C.	Male	31
J.C.	Male	25
L.C.	Female	23
N.C.	Female	26
R.C.	Female	34
R.F.	Male	24
J.H.	Male	22
W.H.	Male	24
K.M.	Female	23
L.M.	Female	33
M.M.	Male	29
D.R.	Male	32
J.S.	Female	25
N.S.	Male	27
P.V.	Female	33
Ph.V.	Male	34

* Referred to as "Controls" in succeeding tables and discussion.

TABLE II

PARENTS OF TRISOMY 21 CHILDREN*

Father	Age	Mother	Age
T.D.	24	D.D.	22
P.H.	26	S.H.	23
S.S.	29	L.S.	29
K.M.	36	G.M.	36

* Referred to as "Parents" in succeeding tables and discussion.

racial differences in mean and modal number of Ag-NOR per cell, but since other parameters have not yet been examined, this study included only Caucasians. The control population could be used in the future to study racial differences. Each couple in the parent group had had one child with Trisomy 21 and one of the mothers, S.H., also had a brother with Trisomy 21. There was no family history of Down's Syndrome in 17 of the controls; this was carefully checked after it was discovered that one female control, L.A., had a nephew with Trisomy 21. Unfortunately, it has not been possible to study the child's mother and compare her data with that of her sister. Parameters were evaluated with and without L.A.'s data; when the exclusion made a difference, it was noted. Every subject studied proved to be karyotypically normal with no apparent mosaicism; one control, K.M., carried a pericentric inversion of

one of her number nines, but this should have no effect on the NORs and their association values.

One of the more general parameters evaluated was a simple calculation of each subject's association frequency (Tables III and IV); this was computed by dividing the number of cells containing at least one association complex by the total number of cells observed, which was, in this study, 50 cells on each individual. If stalk associations are responsible for nondisjunction, higher association rates would be expected to increase the chances of nondisjunction, and the parents would then be expected to have higher rates. The range of association rates in the controls was wide; from 44% to 84% of the cells counted contained an association complex and the entire group had a mean value of 35.1 ± 5.6. All ages and both sexes had values throughout the range. The parents which contained both mothers and fathers showed association rates from 62% to 94%, although only one mother, S.H., had a 90% rate. Otherwise, the highest rate would have been 86% which was barely above the highest control value. The parents' mean rate of 40.0 ± 5.3 was an increase over the controls and, therefore, offered some support for the hypothesis that stalk associations are a mechanism of nondisjunction.

All cells having association complexes were divided by the exact number of complexes contained. It was most common in either group to see cells with one complex. The cells of some individuals, however, were more likely to contain two association groups. It was uncommon to find three complexes in a mitosis, rare to

TABLE III

NOR ASSOCIATIONS OF HUMAN CHROMOSOMES 13,14,15,21,22
IN METAPHASE PLATES OBTAINED FROM
LEUKOCYTE CULTURES

NUMBER OF MITOSES WITH ASSOCIATION COMPLEXES
IN CONTROLS

(50 Mitoses Counted
Per Individual)

Control	1 or More	0	1	2	3	4
L.A.	31	19	21	10	0	0
A.B.	35	15	18	14	2	1
D.C.	27	23	20	7	0	0
J.C.	40	10	22	15	2	1
L.C.	38	12	25	12	1	0
N.C.	32	18	26	6	0	0
R.C.	40	10	25	12	2	1
R.F.	39	11	22	14	1	2
J.H.	38	12	24	11	3	0
W.H.	40	10	20	14	6	0
K.M.	30	20	22	7	1	0
L.M.	22	28	15	6	1	0
M.M.	39	11	17	19	3	0
D.R.	41	9	21	16	4	0
J.S.	35	15	21	10	4	0
N.S.	30	20	19	9	2	0
P.V.	32	18	28	4	0	0
Ph.V.	42	8	22	15	4	1

Mean (1 or more associations) 35.1	Variance 31.0	S.D. ± 5.6
Mean (1 association) 21.6	Variance 10.6	S.D. ± 3.3
Mean (2 association) 11.2	Variance 16.9	S.D. ± 4.1
Mean (2,3,4 associations) 13.5	Variance 31.0	S.D. ± 5.6

TABLE IV

NOR ASSOCIATIONS OF HUMAN CHROMOSOMES 13,14,15,21,22
IN METAPHASE PLATES OBTAINED FROM
LEUKOCYTE CULTURES

NUMBER OF MITOSES WITH ASSOCIATION COMPLEXES
IN PARENTS

(50 Mitoses Counted
Per Individual)

Parent	1 or More	0	1	2	3	4
T.D.	43	7	28	15	0	0
D.D.	34	16	19	13	2	0
P.H.	38	12	23	10	5	0
S.H.	47	3	15	22	9	1
S.S.	42	8	19	20	3	0
L.S.	41	9	30	9	2	0
K.M.	31	19	20	8	3	0
G.M.	44	6	17	22	5	0

Mean (1 or more associations) 40.0	Variance 28.6	S.D. ± 5.3
Mean (1 association) 21.4	Variance 27.7	S.D. ± 5.3
Mean (2 association) 14.9	Variance 33.8	S.D. ± 5.8
Mean (2,3,4 associations) 18.6	Variance 60.6	S.D. ± 7.8

observe four, and no cell from a control or parent was discovered with the theoretically maximum number of five complexes. The parents tended to have more cells with multiple association complexes, but none of the increases seen was statistically significant when tested with an unpaired t test.

The tendency emerged for one or both parents of a child with Trisomy 21 to have an association frequency on the upper end of the scale. The difference between the means for the two groups was not significantly different when the values were subjected to an unpaired t test. Although the highest rate observed was in the cells from a parent and the lowest in the cells of a control, one of the controls who has two normal children had an association rate of 82%. Because of the insignificant difference in the means and the substantial overlap of individual association rates between the two groups, it was concluded that this parameter was of no use as a screening device.

A more exact parameter would be the number of times a specific chromosome pair is involved in an association complex. A high percentage of associations would not necessarily put someone at an increased risk for having a child with Trisomy 21 if the association value for 21, itself, was extremely low. Tables V and VI list the number of times each chromosome pair was involved in an association complex for each individual and for each group under consideration. Every complex was analyzed, and each chromosome which appeared in it was counted once, regardless of the number of chromosomes to which it was connected. If both homologues appeared,

TABLE V

NOR ASSOCIATIONS OF HUMAN CHROMOSOMES 13,14,15,21,22
IN METAPHASE PLATES OBTAINED FROM
LEUKOCYTE CULTURES

FREQUENCY OF CHROMOSOMES IN ASSOCIATION
IN CONTROLS

(50 Mitoses Counted
Per Individual)

Control	13	14	15	21	22
L.A.	14	15	17	32	12
A.B.	21	33	24	16	33
D.C.	14	0	13	24	20
J.C.	23	6	37	43	25
L.C.	15	16	19	36	31
N.C.	16	4	23	21	19
R.C.	29	35	31	22	15
R.F.	26	21	28	32	25
J.H.	20	38	24	24	16
W.H.	19	39	30	26	30
K.M.	12	16	22	21	12
L.M.	5	14	14	24	8
M.M.	7	28	27	41	45
D.R.	37	11	26	19	43
J.S.	30	13	18	25	38
N.S.	21	11	21	17	24
P.V.	12	13	17	5	30
Ph.V.	36	34	24	20	40
Total	357	347	415	448	466
Males	203	188	230	246	268
Females	154	159	185	202	198

X^2 Calculations for Table V

X^2 for Controls

	13	14	15	21	22
0	357	347	415	448	466
E	407	407	407	407	407
0-E	50	60	8	41	59
$\frac{(0-E)^2}{E}$	6.14	8.85	.16	4.13	8.55

$\Sigma^2 = 27.83$

$P < .005$ at 4 D.F.

X^2 for Males

	13	14	15	21	22
0	203	188	230	246	268
E	227	227	227	227	227
0-E	24	39	3	19	41
$\frac{(0-E)^2}{E}$	2.54	6.70	.04	1.59	7.41

$\Sigma^2 = 18.28$

$P < .005$ at 4 D.F.

X^2 for Females

	13	14	15	21	22
0	154	159	185	202	198
E	179	179	179	179	179
0-E	25	20	6	23	19
$\frac{(0-E)^2}{E}$	3.49	2.23	.20	2.96	2.02

$\Sigma^2 = 10.9$

$.05 > P > .025$ at 4 D.F.

then both were counted, but no attempt was made to distinguish between homologues.

The chromosome frequency order from Table V was:

Controls:	22>21>15>13>14
Males:	22>21>15>13>14
Females:	21>22>15>14>13

There is no reason to expect that one chromosome would be involved in associations more frequently than any other at least in a general population large enough for individual characteristics to compensate for one another. It is reasonable, however, to expect an increased frequency of associations involving the number 21 to appear in the parent group, at least in one of a couple.

When chromosome association counts on all controls were totalled, it was obvious that the five pairs were not appearing in approximately equal numbers. More than 100 counts separated the most frequently appearing chromosome number 22 and the least often observed number 14. A chi-square (X^2) test was applied to the counts to test the significance of the deviation from expected values. With high significance ($P < .005$), numbers 22 and 21 were participating in association more often than expected while 15 associated about as expected, and numbers 13 and 14 were not as frequently observed as was expected. When 22 was compared with the sum of 13,14,15, and 21, it still associated significantly more often ($P < .005$) than expected while all other chromosomes associated significantly less often. If 21 was compared in a similar

manner, the significance was still present (.025 > P > .01) but not to the same degree.

Since some previous studies have indicated that females were responsible for the non-randomness of the control sample, the values obtained from males and females were considered separately. The analysis on the male data showed the same frequency order, 22>21>15>13>14, as was observed for all controls; the same highly significant deviation from expected values was also observed. When the female data were analyzed, however, the pattern of chromosome participation in association was not as non-random as was seen in the male controls. The deviation was only significant at .05 > P > .025. The change in the frequency order should be noted; numbers 21 and 22 exchanged positions, but there were only four observations separating the two so that the individual X^2 values were not very different. The order of 14 and 13 was also reversed, and again very few counts separated them. The results on this particular population are different from those samples studied by Hansson and Taysi, but this does not mean that one set of results is right and the other wrong. It emphasizes the fact that randomness and non-randomness of chromosome associations are a characteristic of the individual, and that the population results are a factor of the results on each person studied.

To emphasize this point, X^2 scores were calculated for each individual.

L.A.	21>15>14>13>22	Σ^2 = 14.34	.025 > P > .01
A.B.	22=14>15>13>21	Σ^2 = 9.04	NS
D.C.	21>22>13>15>14	Σ^2 = 23.78	P < .005
J.C.	21>15>22>13>14	Σ^2 = 30.25	P < .005
L.C.	21>22>15>14>13	Σ^2 = 15.74	.01 > P > .005
N.C.	15>21>22>13>14	Σ^2 = 13.30	.025 > P > .01
R.C.	14>15>13>21>22	Σ^2 = 9.70	NS
R.F.	21>15>13>22>14	Σ^2 = 2.53	NS
J.H.	14>15=21>13>22	Σ^2 = 11.51	.05 > P > .025
W.H.	14>15=22>21>13	Σ^2 = 7.27	NS
K.M.	15>21>14>13=22	Σ^2 = 5.41	NS
L.M.	21>14=15>22>13	Σ^2 = 16.31	.01> P > .005
M.M.	22>21>14>15>13	Σ^2 = 29.59	P < .005
D.R.	22>13>15>21>14	Σ^2 = 25.07	P < .005
J.S.	22>13>21>15>14	Σ^2 = 15.48	.01> P > .005
N.S.	22>13=15>21>14	Σ^2 = 5.32	NS
P.V.	22>15>14>13>21	Σ^2 = 22.81	P < .005
Ph.V.	22>13>14>15>21	Σ^2 = 9.19	NS

Seven persons showed a random pattern of chromosome associations which was masked when their data were compiled with the others. Three were female, and four were male, and all age ranges were represented. To further underline individual differences, it can be noted that no one had exactly the same frequency order. Frequency orders are partially a function of which chromosomes carry active NORs. For instance, one control rarely had active NORs on either 14 and therefore had no 14s associating, whereas someone who had a modal number of 10 NORs per cell showed a random pattern of associations. It remains to be seen if some other factor is superimposed on the active NORs in determining NOR associations.

Since L.A. had an excessive number of 21s in association and also had a nephew with Trisomy 21, her values for each chromosome were discarded from the female sample; the frequency order changed from 21>22>15>14>13 to 22>21>15>14>13 where the order of 21 and 22 were in agreement with all males and all controls. It also reduced

the significance of the female X^2 total sufficiently to give a random pattern for associations. Discarding the values from the controls did not change any results except that 21 did not contribute as much to the overall X^2. With this difference noted, L.A.'s values were then left in the controls and females for the duration of the study, because her 21s did not associate any more frequently than those of L.M. who had had a normal child.

Unlike the control group, chromosome number 21 was the most frequently associating chromosome in the parent group. The chromosome frequency order from Table VI was:

Parents:	21>22>15>13>14
Fathers:	21>22>15>13>14
Mothers:	21>22>13>14>15

X^2 calculations indicated that the deviation of number 21 above the expected frequency accounted for approximately 2/3 of the total X^2. When 21 was compared with the total associations of 13, 14, 15, and 21, the X^2 was still highly significant. Although 22 was observed more often than expected, it was not significant when compared with the total of 13, 14, 15, and 21. This contrasted with the control results. Number 15 associated a little less than expected while the association rates for 13 and 14 were substantially decreased.

It is assumed that the supernumerary chromosome is most often present in the egg cell before fertilization and not in the sperm cell. Since it might be assumed that the high association rate for 21 in the parent group was mainly a factor of the mothers, values on both the mothers and fathers were tested separately. It was

TABLE VI

NOR ASSOCIATIONS OF HUMAN CHROMOSOMES 13,14,15,21,22
IN METAPHASE PLATES OBTAINED FROM
LEUKOCYTE CULTURES

FREQUENCY OF CHROMOSOMES IN ASSOCIATION
IN PARENTS

(50 Mitoses Counted
Per Individual)

Parent	13	14	15	21	22
T.D.	29	12	27	48	21
D.D.	14	13	14	39	37
P.H.	8	18	27	41	32
S.H.	43	20	38	50	51
S.S.	21	20	33	35	39
L.S.	21	34	19	42	6
K.M.	16	23	8	24	25
G.M.	29	38	27	43	36
Total	181	178	193	322	247
Fathers	74	73	95	148	117
Mothers	107	105	98	174	130

X^2 Calculations for Table VI

	X^2 for Parents				
	13	14	15	21	22
0	181	178	193	322	247
E	224	224	224	224	224
O-E	43	46	31	98	23
$\frac{(O-E)^2}{E}$	8.25	9.45	4.29	42.86	2.36

$\Sigma^2 = 67.21$

$P < .005$ at 4 D.F.

	X^2 for Fathers				
	13	14	15	21	22
0	74	73	95	148	117
E	101	101	101	101	101
O-E	27	28	6	47	16
$\frac{(O-E)^2}{E}$	7.22	7.76	.36	21.87	2.53

$\Sigma^2 = 39.74$

$P < .005$ at 4 D.F.

	X^2 for Mothers				
	13	14	15	21	22
0	107	105	98	174	130
E	123	123	123	123	123
O-E	16	18	25	51	7
$\frac{(O-E)^2}{E}$	2.08	2.63	5.08	21.15	.40

$\Sigma^2 = 31.34$

$P < .005$ at 4 D.F.

discovered that the 21 participated in associations just as frequently in the fathers as in the mothers. The only difference between the two sets of parents was in the placement of the 15 in the frequency order. Furthermore, every individual had their 21 as either first or second most frequently associating chromosome.

T.D.	21>13>15>22>14 $\Sigma^2 = 26.14$	$P < .005$
D.D.	21>22>13=15>14 $\Sigma^2 = 31.04$	$P < .005$
P.H.	21>22>15>14>13 $\Sigma^2 = 25.88$	$P < .005$
S.H.	22>21>13>15>14 $\Sigma^2 = 16.86$	$P < .005$
S.S.	22>21>15>13>14 $\Sigma^2 = 9.86$	$.05 > P > .025$
L.S.	21>14>13>15>22 $\Sigma^2 = 32.59$	$P < .005$
K.M.	22>21>14>12>15 $\Sigma^2 = 10.89$	$.05 > P > .025$
G.M.	21>14>22>13>15 $\Sigma^2 = 4.98$	NS

This suggested a mechanism intermediate between meiotic errors in germ cells and mitotic errors resulting in mosaicism. Mitotic errors do occur in the blastula and embryo, resulting in an individual with a karyotype of 46/47, +21; the percentage of trisomic cells depends on how early the nondisjunction occurred. Since this is possible, it is also conceivable that nondisjunction of number 21 could occur at the first cell division in the zygote. If this were to happen, one cell would be monosomic for and one cell would

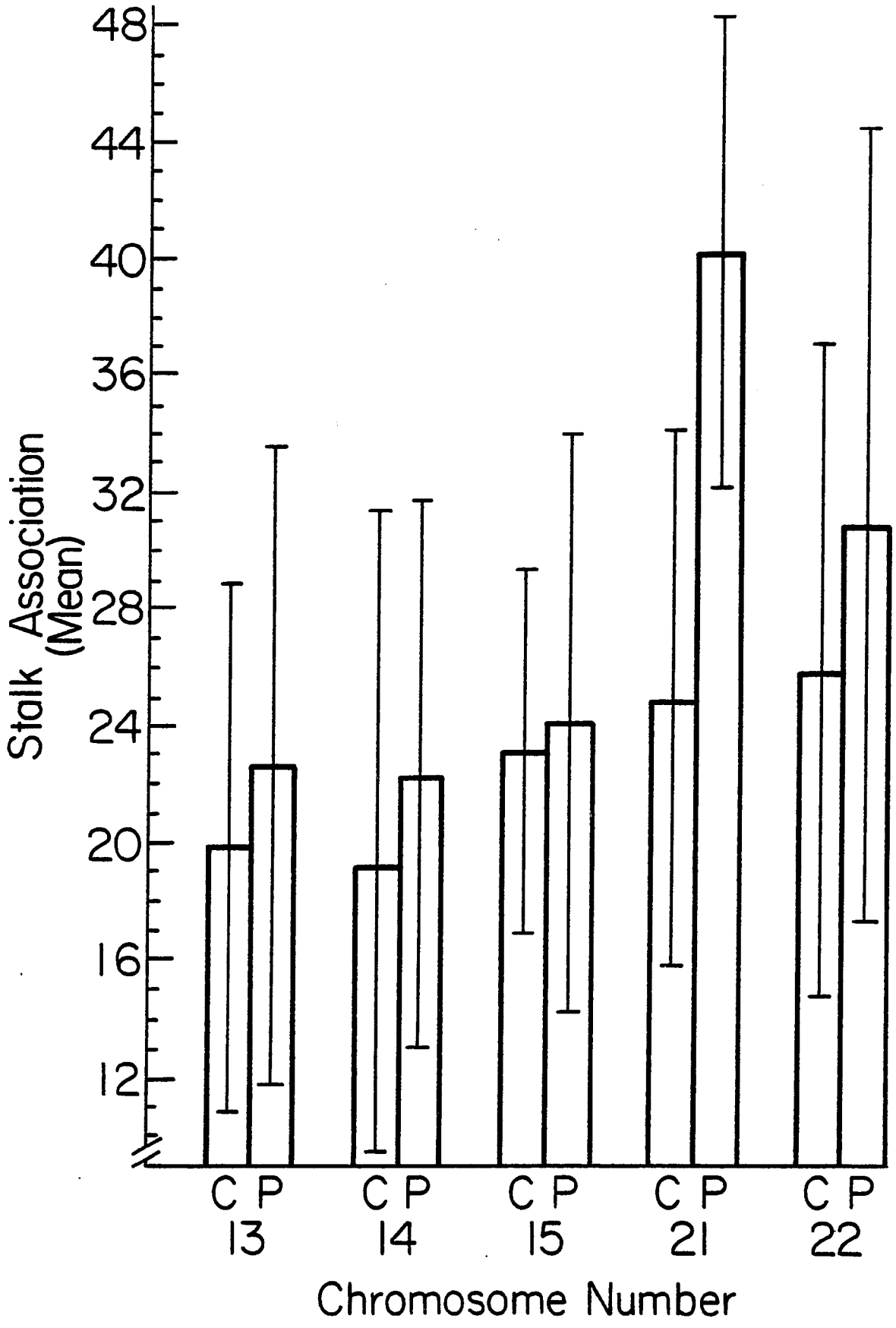
be trisomic for 21. Monosomy of an autosome is generally considered lethal; therefore, the monosomic cell would probably die, leaving one viable cell with an extra 21. This cell would then continue to divide to produce an embryo and later a fetus with the classical Down's Syndrome. There would be no way to distinguish this child from one who had developed from an imperfect egg or sperm. If each parent has high association rates for both 21s, the child would receive a 21 from each parent, both of which associate quite readily. It would then be possible for high association rates to contribute to trisomy 21 in the zygote, not the egg or sperm. It now seems that there may be three occasions for an error to occur but the results of two instances would be the same.

The tendency toward increased association of the number 21 has been established in the parents of children with Trisomy 21. The mean scores of each chromosome in both the control and test groups were calculated and graphed (Figure 4) to determine if there was an increased frequency for all chromosomes in the parents or if 21 was a special case.

Mean Score for Each Chromosome

13	Controls:	19.8 ± 9.1
	Parents:	22.6 ± 10.9
14	Controls:	19.2 ± 12.2
	Parents:	22.3 ± 9.3
15	Controls:	23.1 ± 6.2
	Parents:	24.1 ± 9.9

Figure 4. Mean number of stalk associations in parents and in controls for chromosome numbers 13, 14, 15, 21, and 22.


48
44
40
36
32
28
24
20
16
12
Stalk Association (Mean)
C P
13
C P
14
C P
15
C P
21
C P
22
Chromosome Number

21	Controls:	24.9 ± 9.2
	Parents:	40.3 ± 8.1
22	Controls:	25.9 ± 11.2
	Parents:	30.9 ± 13.6

The association values for 13-15 and 22 were very similar in the two groups, but the mean scores for 21 were quite different. An unpaired t test was applied to test for any significance, and it was discovered that only the number 21 associated significantly more often in the parents than in the controls ($P < .001$). This may be taken as evidence that stalk associations do play a role in nondisjunction, but the individual scores of a few of the controls are too similar to the parent group to use this as a definite method of screening.

A question which has been raised in the past concerns the pairing of chromosomes in association complexes; some pairs appear to be more common than others. Tables VII and VIII list the chromosome combinations by groups. Two chromosome complexes were by far the most common: 84% of all complexes in the controls and 80% of all complexes in the parents were composed of either a D and a G, two Ds, or two Gs. On the basis of the number of chromosomes available for association, 53.3% of all two chromosome pairings should be D-G combinations, 33.3% should be D-D pairs, and 13.3% should be G-G associations. A normal distribution of pairs would be expected in the controls as a whole, but it can be hypothesized

TABLE VII

DISTRIBUTION OF CHROMOSOME COMBINATIONS
WHICH MAKE UP ASSOCIATION COMPLEXES
IN CONTROLS

Control	D-G	D-D	G-G	D-D-G	D-G-G
L.A.	22	8	6	1	2
A.B.	26	12	6	5	2
D.C.	17	4	10	0	2
J.C.	32	12	8	3	4
L.C.	25	5	12	2	4
N.C.	13	9	9	2	2
R.C.	25	25	2	1	1
R.F.	29	15	9	1	3
J.H.	18	20	5	4	3
W.H.	27	20	9	5	2
K.M.	17	12	5	1	1
L.M.	19	5	1	0	4
M.M.	23	11	13	2	10
D.R.	35	14	11	4	0
J.S.	29	7	6	3	4
N.S.	21	11	6	2	1
P.V.	19	8	4	3	1
Ph.V.	28	21	6	4	4
Total	425	219	128	43	50

TABLE VII (Continued)

					(50 Mitoses Counted Per Individual)
D-D-D	G-G-G	D-D-G-G	D-D-D-G	D-G-G-G	Other
0	0	1	0	0	1
3	0	0	1	0	1
0	1	0	0	0	0
0	3	0	0	0	0
1	1	1	0	0	1
2	1	0	0	0	0
0	0	1	1	0	3
1	0	0	1	1	1
4	1	0	0	0	0
1	0	1	0	0	0
2	1	0	0	0	0
0	1	0	0	0	0
0	2	1	0	1	1
0	0	1	0	0	0
0	1	0	1	0	2
0	0	0	1	0	1
0	1	0	0	0	0
1	0	2	1	0	1
15	13	8	6	2	12

X^2 Calculations for Table VII

X^2 for Controls

	D-G	D-D	G-G
O	425	219	128
E	412 (53.3%)	257 (33.3%)	103 (13.3%)
O-E	13	38	25
$\frac{(O-E)^2}{E}$	.41	5.62	6.07

$\Sigma^2 = 12.1$

$P < .005$ at 2 D.F.

X^2 for Males

	D-G	D-D	G-G
O	230	128	77
E	232 (53.3%)	145 (33.3%)	58 (13.3%)
O-E	2	17	19
$\frac{(O-E)^2}{E}$	.02	1.99	6.22

$\Sigma^2 = 8.23$

$.025 > P > .01$ at 2 D.F.

X^2 for Females

	D-G	D-D	G-G
O	195	91	51
E	180 (53.3%)	112 (33.3%)	45 (13.3%)
O-E	15	21	6
$\frac{(O-E)^2}{E}$	1.25	3.94	.80

$\Sigma^2 = 5.99$

NS at 2 D.F.

TABLE VIII

DISTRIBUTION OF CHROMOSOME COMBINATIONS
WHICH MAKE UP ASSOCIATION COMPLEXES
IN PARENTS

Parent	D-G	D-D	G-G	D-D-G	D-G-G
T.D.	26	9	8	4	5
D.D.	13	5	20	2	7
P.H.	24	7	17	3	3
S.H.	43	13	17	7	4
S.S.	34	12	13	3	2
L.S.	26	14	3	3	4
K.M.	26	6	8	3	1
G.M.	30	18	10	9	4
Total	222	84	96	34	30

TABLE VIII (Continued)

				(50 Mitoses Counted Per Individual)	
D-D-D	G-G-G	D-D-G-G	D-D-D-G	D-G-G-G	Other
1	1	1	0	1	2
1	1	2	0	0	0
2	2	0	0	0	0
1	1	2	0	0	2
0	1	1	2	0	0
2	0	1	0	0	1
0	0	1	0	0	0
0	2	1	1	1	0
7	8	9	3	2	5

X^2 Calculations for Table VIII

X^2 for Parents

	D-G	D-D	G-G
0	222	84	96
E	214 (53.3%)	134 (33.3%)	54 (13.3%)
0-E	8	50	42
$\frac{(0-E)^2}{E}$	.30	18.66	32.67

$\Sigma^2 = 51.63$

$P < .005$ at 2 D.F.

X^2 for Fathers

	D-G	D-D	G-G
0	110	34	46
E	101 (53.3%)	63 (33.3%)	25 (13.3%)
0-E	9	29	21
$\frac{(0-E)^2}{E}$	.80	13.35	17.64

$\Sigma^2 = 31.79$

$P < .005$ at 2. D.F.

X^2 for Mothers

	D-G	D-D	G-G
0	112	50	50
E	113 (53.3%)	71 (33.3%)	28 (13.3%)
0-E	1	21	22
$\frac{(0-E)^2}{E}$	.01	6.21	17.30

$\Sigma^2 = 23.50$

$P < .005$ at 2 D.F.

that the test group would exhibit more D-G and G-G pairs than D-D pairings. The frequency order of two chromosome pairs derived from Table VII was as expected:

Controls:	D-G>D-D>G-G
Males:	D-G>D-D>G-G
Females:	D-G>D-D>G-G

It can be seen, however, from the X^2 tables that even in the controls, the G-G pairs were more frequently observed than was expected; the D-D pairings were not as common as expected. This non-randomness was seen in the controls as a whole and in the males, whereas the females were seen to have a random pattern of chromosome pairing.

The frequency order for two chromosome pairs in the parent group did differ from that seen in the controls.

Parents:	D-G>G-G>D-D
Fathers:	D-G>G-G>D-D
Mothers:	D-G>G-G=D-D

D-G pairs were still the most common, but G-G combinations were more frequently seen than D-D pairs. Five of the parents individually showed an increase of G-G over D-D, and none of them showed an increase of D-D over D-G as was seen in one of the controls. The non-random pairings seen in the parents as a whole was also seen in both the mothers and fathers when their values were figured separately.

An increase in G-G association pairs was observed in both the parents and controls. An unpaired t test was applied to the

parental mean and the control mean to determine if there were significantly more G-G associations in the mitoses of the parents. Significance was indeed found at the .02 level (.02 > P > .01). The parents would also be expected to have a higher number of association complexes with more than two chromosomes. When values in the last eight columns of Tables VII and VIII were totaled and the mean scores calculated, the controls had an average of 8.28 complexes containing three or more chromosomes while the parents had an average value of 12.25 complexes per 50 metaphase spreads. The difference was significant at the .05 level (.05 > P > .02). In general, the parents of children with Trisomy 21 were more likely to have a significant increase in the number of G-G associations and in the number of association complexes which contained more than two chromosomes. This agrees well with what was found in the satellite association studies. Unfortunately, there was too great an overlap in the control and parent ranges to make this parameter useful in identifying those at an increased risk for having a child with a trisomy.

Techniques for banding chromosomes made it possible to study specific chromosome pairs which have been listed in Tables IX and X. The D-G, D-D, and G-G pairs (from Tables VII and VIII) were all listed in the more specific columns while association complexes containing more than two chromosomes were divided into n number of pairs depending on the morphology of the complex. If a chromosome was attached to two other chromosomes, for instance, it was counted as part of two pairs; it was usually not hard to determine which

TABLE IX

PAIR ASSOCIATIONS OF CHROMOSOMES 13,14,15,21,22
IN CONTROLS

Control	13-21	14-21	15-21	22-21	21-21	13-22	14-22
L.A.	9	7	8	4	5	3	2
A.B.	2	4	5	5	0	7	12
D.C.	5	0	7	8	3	6	0
J.C.	6	5	17	11	4	4	1
L.C.	7	5	9	13	3	4	8
N.C.	4	2	4	11	1	3	0
R.C.	7	7	8	2	1	7	5
R.F.	6	4	9	7	6	7	4
J.H.	4	9	3	7	2	1	6
W.H.	5	6	5	6	3	7	9
K.M.	4	4	6	6	2	1	2
L.M.	2	8	10	3	2	0	3
M.M.	3	9	6	21	8	2	13
D.R.	5	4	5	6	0	14	3
J.S.	11	4	1	8	3	12	6
N.S.	7	2	0	7	0	4	4
P.V.	0	1	0	5	0	7	5
Ph.V.	9	5	5	7	0	15	10
Total	96	86	108	137	43	104	93

Mean (21-22 pairs)	7.6	Variance 18.8	S.D. ± 4.3
Mean (21-21 pairs)	2.4	Variance 5.2	S.D. ± 2.3
Mean (all pairs containing 21)	26.1	Variance 95.2	S.D. ± 9.8

TABLE IX (Continued)

(50 Mitoses Counted Per Individual)

15-22	22-22	13-14	13-15	14-15	13-13	14-14	15-15
5	0	3	2	3	0	0	0
7	3	10	6	7	1	3	1
2	3	0	4	0	0	0	0
8	2	0	9	0	3	0	3
7	3	2	4	3	0	0	0
8	1	2	8	0	0	0	3
5	0	10	6	11	2	3	3
8	1	5	9	6	0	2	1
4	0	11	9	8	0	5	1
6	2	4	5	16	0	5	0
3	0	1	5	7	1	1	1
2	0	2	1	2	0	0	0
16	5	2	0	6	0	2	1
11	6	3	10	1	3	0	0
9	5	2	9	2	0	0	0
12	2	4	5	2	1	0	3
11	3	4	1	4	0	0	1
5	6	10	4	7	2	2	2
129	42	75	97	85	13	23	20

TABLE X

PAIR ASSOCIATIONS OF CHROMOSOMES 13,14,15,21,22
IN PARENTS

Parent	13-21	14-21	15-21	22-21	21-21	13-22	14-22
T.D.	14	4	14	8	9	6	2
D.D.	4	5	7	19	5	9	4
P.H.	4	5	12	16	5	1	5
S.H.	13	5	12	16	5	15	7
S.S.	4	9	9	15	1	8	5
L.S.	4	19	10	2	5	0	4
K.M.	5	10	5	6	0	7	8
G.M.	9	13	9	19	1	5	9
Total	62	70	78	101	31	51	44

Mean (21-22 pairs)	12.6	Variance 41.1	S.D. ± 6.4
Mean (21-21 pairs)	3.9	Variance 9.0	S.D. ± 3.0
Mean (all pairs containing 21)	42.8	Variance 70.2	S.D. ± 8.4

TABLE X (Continued)

(50 Mitoses Counted Per Individual)

15-22	22-22	13-14	13-15	14-15	13-13	14-14	15-15
8	3	5	6	3	2	0	0
3	4	2	2	3	0	0	1
6	2	3	4	7	0	0	1
13	2	7	10	3	3	0	3
11	2	4	5	4	1	0	4
1	0	8	3	8	2	1	0
2	4	5	2	1	0	1	0
9	1	10	4	9	2	1	0
53	18	44	36	38	10	3	9

X^2 Comparison of Tables IX and X

Controls	13-21	14-21	15-21	22-21	21-21	13-22	14-22
O	96	86	108	137	43	104	93
E	102	102	102	102	26	102	102
O-E	6	16	6	35	17	2	9
$\frac{(O-E)^2}{E}$	.35	2.51	.35	12.01	11.12	.04	.79

$\Sigma^2 = 62.63$

$P < .005$ at 14 D.F.

Parents	13-21	14-21	15-21	22-21	21-21	13-22	14-22
O	62	70	78	101	31	51	44
E	58	58	58	58	14	58	58
O-E	4	12	20	43	17	7	14
$\frac{(O-E)^2}{E}$	.28	2.48	6.90	31.88	20.64	.84	3.38

$\Sigma^2 = 98.16$

$P < .005$ at 14 D.F.

X^2 Comparison of Tables IX and X (Continued)

15-22	22-22	13-14	13-15	14-15	13-13	14-14	15-15
129	42	75	97	85	13	23	20
102	26	102	102	102	26	26	26
27	16	27	5	17	13	3	6
7.15	9.85	7.15	.25	2.83	6.50	.35	1.38

15-22	22-22	13-14	13-15	14-15	13-13	14-14	15-15
53	18	44	36	38	10	3	9
58	14	58	58	58	14	14	14
5	4	14	22	20	4	11	5
.43	1.14	3.38	8.34	6.90	1.14	8.64	1.79

NORs were associating. There are 15 possible pairs, five of which are homologous. Theoretically, the non-homologous pairs should be observed four times more often than the homologous ones. For both the control and the test groups, the frequency order placed all ten unlike pairs above the five homologous combinations.

Controls	Parents
21-22	21-22
15-22	15-21
15-21	14-21
13-22	13-21
13-15	15-22
13-21	13-22
14-22	14-22
14-21	13-14
14-15	14-15
13-14	13-15
21-21	21-21
22-22	22-22
14-14	13-13
15-15	15-15
13-13	14-14

The most frequent pairing in either group was between chromosomes 21 and 22; previous studies (Taysi, 1975; Hansson, 1979; Galperin-Lemaître et al., 1980) have also found this to be true. For the remainder of the non-homologous pairs, 13-21, 14-21, 15-21, all followed 22-21 as the next most common pairings in the parents. These three pairs were ranked between three and eight in the frequency order for the controls. Among the homologous pairs, both pairs involving Gs were ranked above the three D-D pairs in rate of occurrence with 21-21 as the most common pairing in both parents and controls. A Wilcoxin rank sum test was performed on these rankings of the frequency orders (page 66) to determine if a significant difference existed between parents and controls. It was

found that the probability of 13-21, 14-21, 15-21, 21-22, and 21-21 being ranked as they were in the controls was 1 in 6. The probability that the four non-homologous pairs in the parents would be ranked from one through four while 21-21 was ranked first among the homologous pairs was 1 in 1050. The difference between the parents and controls in the ranking of pairs containing a 21 was highly significant ($P < .001$).

The X^2 calculations indicated that these chromosome pairings were indeed non-random. In the controls, the non-homologous pairs which were observed more often than expected included 21-22, 15-22, 15-21, and 13-22, while the other six occurred less often than expected. Of the homologous pairs, only 21-21 and 22-22 were seen in greater numbers than expected. It should be noted that 21 and 22 were almost equally represented among the most commonly occurring pairs. Among the parents, all five pairs containing a 21 and the pair 22-22 were observed more often than expected. When X^2 scores were calculated on the total number of pairs including a 21 versus all other pairs, the deviation from the expected values was highly significant ($P < .005$). In other words, the non-randomness in the control group was due to the excess number of pairs involving both the 21 and 22 while the non-randomness found among the parents was because of the extremely large number of pairs involving the 21st chromosomes.

Unpaired t tests were used to compare certain mean values in the controls with those in the parents. There were significantly more 21-22 pairs in the parents ($0.5 > P > .02$), but the higher

X^2 Calculation from Table IX

Controls	Pairs Including a 21	All Other Pairs
O	470	681
E	434	716
O-E	36	35
$\frac{(O-E)^2}{E}$	2.99	1.71

$\Sigma^2 = 4.70$

NS

X^2 Calculation from Table X

Parents	Pairs Including a 21	All Other Pairs
O	342	306
E	246	404
O-E	96	98
$\frac{(O-E)^2}{E}$	37.46	23.77

$\Sigma^2 = 61.23$

$P < .005$ at 2 D.F.

number of 21-21 pairs in this group was not significantly different from that in the controls. Since all pairs containing a 21 were seen more often than expected in the parents, a t test was run on the mean of these to determine if the parents were more likely than the controls to have pairs involving the 21. The total number of 13-21, 14-21, 15-21, 21-21, and 22-21 pairs was significantly higher among the parents as opposed to controls at the .001 level. It would not be possible, however, to predict an individual's chances of having a child with Trisomy 21 from this parameter because of the overlap between the ranges of the controls and parents.

Hansson (1979) determined that there was a significant difference in the number of association complexes per cell and the number of chromosomes per complex in parents of Trisomy 21 children as opposed to controls. Tables XI and XII list the average number of complexes found in the subjects of this study; an average was determined by dividing the total number of groups in 50 cells by 50. The controls ranged anywhere from .60 to 1.36, and the parents ranged from .90 to 1.80. While it was not uncommon to find the average number of complexes in a control below 1.0 per cell, only one of the parents had an average this low. The two highest averages found were in the parents; this raised the mean to 1.25. A t test was applied to this and to the control mean of 1.02 and showed that the difference between the two values was significant at the .05 level. While Zang and Back (1968) and Hansson (1979) did not

TABLE XI

CHROMOSOME COMPLEXES PER METAPHASE PLATE IN CONTROLS

(50 Mitoses Counted Per Individual)

Control	No. of Complexes (Average)
L.A.	.82
A.B.	1.12
D.C.	.68
J.C.	1.24
L.C.	1.04
N.C.	.76
R.C.	1.18
R.F.	1.22
J.H.	1.10
W.H.	1.32
K.M.	.78
L.M.	.60
M.M.	1.28
D.R.	1.30
J.S.	1.06
N.S.	.86
P.V.	.72
Ph.V.	1.36

Mean	1.02	Variance .06	S.D. ± .25
Mean (Males)	1.15	Variance .05	S.D. ± .22
Mean (Females)	.90	Variance .04	S.D. ± .20

TABLE XII

CHROMOSOME COMPLEXES PER METAPHASE PLATE IN PARENTS

(50 Mitoses Counted Per Individual)

Parent	No. of Complexes (Average)
T.D.	1.16
D.D.	1.02
P.H.	1.16
S.H.	1.80
S.S.	1.36
L.S.	1.08
K.M.	.90
G.M.	1.52

Mean	1.25	Variance .09	S.D. ± .30
Mean (Fathers)	1.15	Variance .04	S.D. ± .20
Mean (Mothers)	1.36	Variance .14	S.D. ± .37

find any differences between control males and females, this population showed a significant difference ($.05 > P > .02$). The female mitoses had fewer association complexes than the male cells, but in the parental group, the mothers had a higher average than the fathers. It was then suspected and proven with a t test that there was a significant difference in the average number of chromosome complexes per cell between female controls and mothers of children with Trisomy 21. This is the first parameter where the male controls and fathers have had very similar mean scores which were between the low value for the female controls and the significantly higher value for the mothers. It was unfortunate that the range of values for the two female groups overlapped as it did, otherwise this would have been a possible screening test.

The average number of chromosomes per complex (Tables XIII and XIV) was derived by dividing the total number of chromosomes in association (Tables V and VI) by the total number of complexes (Tables VII and VIII). No significant differences were seen between the parents and controls, between the male and female controls, or between the mothers and fathers. The different criteria for determining association complexes could explain the difference seen in this parameter between these results and those of Hansson. Hansson's work was done without silver staining, and any acrocentrics within a certain distance of one another with the correct orientation of short arm to short arm were considered to be in association. Many instances of two or more chromosomes being so oriented without silver grains connecting the NORs were observed

TABLE XIII

CHROMOSOMES PER ASSOCIATION COMPLEX IN CONTROLS

(50 Mitoses Counted Per Individual)

Control	No. of Chromosomes (Average)
L.A.	2.20
A.B.	2.27
D.C.	2.08
J.C.	2.16
L.C.	2.25
N.C.	2.18
R.C.	2.24
R.F.	2.16
J.H.	2.22
W.H.	2.18
K.M.	2.13
L.M.	2.17
M.M.	2.31
D.R.	2.09
J.S.	2.34
N.S.	2.19
P.V.	2.13
Ph.V.	2.26

Mean	2.20	Variance .007	S.D. ± .08
Mean (Males)	2.18	Variance .009	S.D. ± .09
Mean (Females)	2.21	Variance .005	S.D. ± .07

TABLE XIV

CHROMOSOMES PER ASSOCIATION COMPLEX IN PARENTS

(50 Mitoses Counted Per Individual)

Parent	No. of Chromosomes (Average)
T.D.	2.36
D.D.	2.29
P.H.	2.17
S.H.	2.24
S.S.	2.18
L.S.	2.26
K.M.	2.13
G.M.	2.28

Mean	2.24	Variance .007	S.D. ± .08
Mean (Fathers)	2.21	Variance .01	S.D. ± .1
Mean (Mothers)	2.27	Variance .002	S.D. ± .04

during the course of this study. These were not counted as associations, and there were enough of these instances to possibly make a difference in the results, especially in Table IV.

Silver positive NORs and silver positive connectives between the NORs are prerequisites for the determination of stalk associations. The tendency exists for the parents to have an increased association rate and an increased number of 21s in association. Therefore, it might be expected that the mean number of Ag-NORs and the mean number of G group chromosome Ag-NORs would be increased in the parents. The overall mean of Ag-NORs in the controls was 8.13 ± .89 (Table XV); this was very close to the value obtained by Howell et al. (unpublished data). The modes ranged from 6 to 10 with 8 and 9 being the most common ones observed. There was no significant difference in these values and those found in the parents (Table XVI). The parental group did have higher overall means for all Ag-NORs, all D group Ag-NORs (Tables XVII and XVIII), and all G group Ag-NORs (Tables IXX and XX), but none were statistically significant. This indicated that some other factors were involved in determining association frequencies. For instance, L.M. had a mean of 8.42 which was higher than the overall mean in either the parental or control groups; yet, her association frequency (Table III) was the lowest observed. S.H. consistently had five Ds and four Gs carrying Ag-NORs; a ratio of five Ds to four Gs in association would be expected if no other factors were involved, but an equal number of Ds and Gs were found to be participating in association. All cell culture conditions, harvesting, and slide

TABLE XV

FREQUENCY OF THE SILVER STAINED NUCLEOLUS ORGANIZER REGIONS IN CONTROLS

(50 Mitoses Counted Per Individual)

Control	All Ag + NORs Mean	Median	Mode
L.A.	6.76	7	7
A.B.	8.92	9	9
D.C.	6.22	6	6
J.C.	8.46	9	9
L.C.	8.20	8	8
N.C.	7.42	7	7
R.C.	8.60	9	9
R.F.	9.88	10	10
J.H.	7.88	8	8
W.H.	9.36	10	10
K.M.	8.45	9	9
L.M.	8.42	9	9
M.M.	8.50	9	9
D.R.	7.38	7	7
J.S.	7.90	8	8
N.S.	8.10	8	8-9
P.V.	7.32	7	8
Ph.V.	8.62	9	9

Overall Mean 8.13 Variance .79 S.D. ± .89

TABLE XVI

FREQUENCY OF THE SILVER STAINED NUCLEOLUS ORGANIZER REGIONS IN PARENTS

(50 Mitoses Counted Per Individual)

Parent	All Ag + NORs Mean	Median	Mode
T.D.	7.88	8	8
D.D.	7.52	8	7-8
P.H.	7.78	8	8
S.H.	8.88	9	9
S.S.	8.84	9	9
L.S.	8.34	8	9

Overall Mean 8.21 Variance .33 S.D. ± .57

TABLE XVII

FREQUENCY OF THE SILVER STAINED NUCLEOLUS ORGANIZER REGIONS ON D GROUP CHROMOSOMES IN CONTROLS

(50 Mitoses Counted Per Individual)

Control	D Group Ag + NORs		
	Mean	Median	Mode
L.A.	3.98	4	4
A.B.	5.84	6	6
D.C.	2.36	2	2
J.C.	4.76	5	5
L.C.	4.24	4	4
N.C.	4.38	4	4
R.C.	5.94	6	6
R.F.	5.96	6	6
J.H.	5.06	5	5
W.H.	5.74	6	6
K.M.	5.04	5	5
L.M.	5.06	5	5
M.M.	4.52	5	4-5
D.R.	5.44	4	4
J.S.	4.14	4	4
N.S.	4.58	5	4
P.V.	4.24	4	5
Ph.V.	5.62	6	6

Overall Mean 4.83 Variance .82 S.D. ± .91

TABLE XVIII

FREQUENCY OF THE SILVER STAINED NUCLEOLUS ORGANIZER REGIONS ON D GROUP CHROMOSOMES IN PARENTS

(50 Mitoses Counted Per Individual)

Parent	D Group Ag + NORs		
	Mean	Median	Mode
T.D.	4.70	5	5
D.D.	3.84	4	4
P.H.	4.00	4	4
S.H.	4.92	5	5
S.S.	4.98	5	5
L.S.	5.76	6	6

Overall Mean 4.70 Variance .50 S.D. ± .71

TABLE IXX

FREQUENCY OF THE SILVER STAINED
NUCLEOLUS ORGANIZER REGIONS
ON G GROUP CHROMOSOMES
IN CONTROLS

(50 Mitoses Counted
Per Individual)

Control	G Group Ag + NORs		
	Mean	Median	Mode
L.A.	2.78	3	3
A.B.	3.08	3	3
D.C.	3.86	4	4
J.C.	3.70	4	4
L.C.	3.96	4	4
N.C.	3.02	3	3
R.C.	2.66	3	3
R.F.	3.92	4	4
J.H.	2.82	3	3
W.H.	3.62	4	4
K.M.	3.44	4	4
L.M.	3.40	4	4
M.M.	3.98	4	4
D.R.	2.92	3	3
J.S.	3.76	4	4
N.S.	3.52	4	4
P.V.	3.08	3	3
Ph.V.	3.00	3	3

Overall Mean 3.36 Variance .20 S.D. ± .45

TABLE XX

FREQUENCY OF THE SILVER STAINED
NUCLEOLUS ORGANIZER REGIONS
ON G GROUP CHROMOSOMES
IN PARENTS

(50 Mitoses Counted
Per Individual)

Parent	G Group Ag + NORs		
	Mean	Median	Mode
T.D.	3.18	3	4
D.D.	3.68	4	4
P.H.	3.78	4	4
S.H.	3.96	4	4
S.S.	3.86	4	4
L.S.	2.56	3	3

Overall Mean 3.50 Variance .29 S.D. ± .54

making techniques were uniform for every subject studied. There may be some inherent difference in the NORs which causes one to associate very often and one to remain unassociated in most cells.

Meiosis and mitosis are complex processes of cellular proliferation which occur many times over without an error. When an error does occur either in a gamete or in a zygote, the maldistribution of chromosomes between two daughter cells can have tragic results. Certain chromosomes are more often found in trisomic states which probably resulted from nondisjunction; the reasons and mechanisms behind this are unclear. In fact, there may be more than one mechanism by which a trisomy arises, especially for chromosomes of different morphology. The acrocentric chromosomes have a morphology which allows them to participate in nucleolus formation, and as a result, become involved in physical associations at the nucleolar organizer regions (NORs), which are designated as "stalk associations." Experimental evidence supports the theory that stalk associations may be at least one mechanism behind the occurrence of nondisjunction. Parents of children with Trisomy 21 have been found to have an increased chromosome association rate, but more specifically, the number 21 is involved in association complexes significantly more often in these parents than in a general control population. The total number of chromosome pairs which involve a 21 occurs significantly more often in the parents. These significant increases are superimposed on a non-random pattern of chromosome associations in the general population; it is

possible that this accounts for the high incidence of Down's Syndrome. Number 22 also associates frequently, but Trisomy 22 is rarely seen in the clinical setting. Those cases which have occurred as live births have been much more severely affected than children with Down's Syndrome. Therefore, it is possible that Trisomy 22 occurs as frequently as Trisomy 21, but it is aborted spontaneously in gestation, since it seems to be so incompatible with life. Hassold et al. (1978) have studied the cytogenetics of material from spontaneous abortions and have supported this theory by finding Trisomy 21 and 22 in almost equal proportions.

CHAPTER IV

CONCLUSIONS

Parents of children with Trisomy 21 were observed to have increased stalk association rates over members of a control population; this increase was not statistically significant, however.

Whether chromosomes participate in association in a random or a non-random manner is an individual characteristic.

The association rate of chromosome 21 was significantly increased in the parents as opposed to controls, whereas all other chromosomes associated at approximately the same frequency in each group.

A significant increase of G-G pairs was observed in the parents, and this was superimposed on a general rise in G-G associations at the expense of D-D pairs observed in controls.

Chromosomes were seen to have preferred partners in association complexes, as 21-22 was the most common pairing in both the control and parent groups. There were significantly more 13-21, 14-21, 15-21, 21-21, and 22-21 associating pairs in the parents than in the controls.

A significant increase in the parent's average number of association complexes per cell was observed, but no difference in

the size of the complexes was noted when comparing the control values with the parental values.

The mean and modal numbers of Ag-NORs were very similar in the parents and the controls. Except for the fact that Ag-NORs must be present for stalk associations to form, there does not appear to be any relationship between the frequency of Ag-NORs and the frequency of association complexes.

Evidence supports the hypothesis that association of chromosomes at the Nucleolar Organizer Regions may be a mechanism for nondisjunction. No adequate screening test for couples at high risk for having a child with a trisomy has yet been found.

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